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The Role of the Cleaved Cytoplasmic Domain of E-Cadherin in Signalling

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DECLARATION

I, Emma Clare Ferber, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

ABSTRACT

E-cadherin is the most crucial membrane protein for the formation of tight and compact cell-cell adhesions in epithelial cells. E-cadherin based cell-cell junctions (adherens junctions) are highly dynamically regulated, for example during epithelial to mesenchymal transition (EMT) when E-cadherin at cell-cell contacts is down-regulated, leading to a loss of cell-cell adhesion. In addition to its structural role, E-cadherin has been shown to be involved in several signalling pathways during cell proliferation, differentiation and cell survival. However the molecular mechanisms of E-cadherin-mediated signalling are largely unknown. E-cadherin binding proteins, β-catenin and p120-catenin (p120), can translocate to the nucleus, and bind and regulate the activity of transcription factors TCF/LEF-1 and Kaiso respectively. The influence of E-cadherin in these pathways is not fully understood. E-cadherin has been shown to be cleaved by metalloproteases and also by γ-secretase. In this thesis I have examined E-cadherin cleavage and the role of cleaved E-cadherin in signalling.

The conditions inducing the cleavage of E-cadherin in epithelial cell lines were investigated. It was found that the γ-secretase cleavage product of E-cadherin (E-cad/CTF2) was produced in MDCK cells in response to stimuli leading to EMT: treatment with hepatacyte growth factor and activation of Ras. p120 was shown to specifically enhance the nuclear localisation of E-cad/CTF2. p120 was also found to induce the ubiquitination of E-cad/CTF2. Biochemical assays were used to show that p120 binds to a RING-finger containing protein, ret finger protein (RFP), which also enhances ubiquitination and nuclear translocation of E-cad/CTF2. The role of ubiquitination and phosphorylation of E-cad/CTF2 in its nuclear translocation was investigated. In the nucleus, E-cad/CTF2 was shown to bind specifically to DNA. Its association with DNA is enhanced by both p120 and RFP. The transactivation potential of E-cad/CTF2 was investigated. It was shown that nuclear E-cad/CTF2 regulates β-catenin-TCF/LEF-1- and p120-Kaiso-mediated signalling pathways.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

1 INTRODUCTION

1.1 Cell-cell junctions

In multi-cellular organisms, individual cells are often connected to each other via cell-cell adhesions to form three-dimensionally structured tissues. Formation of tight and compact cell-cell adhesions restricts cell movement and provides cells with a positional cue for the establishment of cell polarity. Different tissues have different requirements for cell-cell adhesion strengths. In connective tissues the extracellular matrix (ECM) is abundant, cells are sparsely distributed, and direct cell-cell contacts are of relatively low importance for the mechanical strength of the tissue. However in epithelial tissues, the cells are tightly bound to each other to form sheets of cells (Figure 1.1). In these epithelial tissues, the extracellular matrix is very sparse and forms a thin barrier, known as the basal lamina, to which the epithelial sheet is bound via cell-matrix adhesions (Figure 1.1). Epithelial cells have a defined polarity, with the apical side facing the the lumen and the basolateral side facing the basal lamina (Figure 1.1).

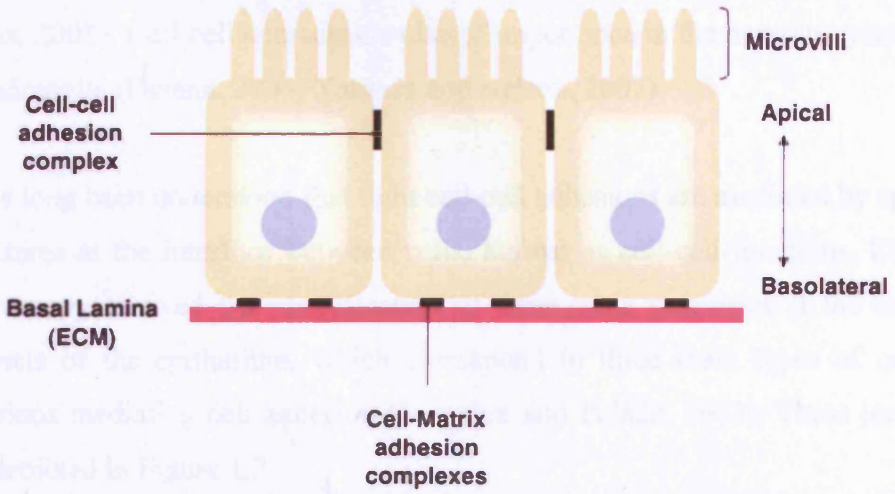

Figure 1.1 ***Epithelial cells in an epithelial sheet.*** *Within an epithelial cell sheet, individual cells are tightly connected to each other via cell-cell adhesion complexes. They are also adhered to the extracellular matrix (ECM), which in epithelial tissue forms a thin basal lamina via cell-matix adhesion complexes such as hemidesmosomes and focal adhesions.*

Epithelial sheets are of vital importance to the organs of animals, lining all the cavities, lumens and free surfaces, including the respiratory, digestive, circulatory and renal tracts, as well as the skin. Epithelial sheets are either simple containing only one layer of highly polarised cells such as the lining of the small intestine as depicted in Figure 1.1, or stratified containing several layers of less polarised cells such as in skin. The basic function of the epithelium is to act as a barrier to the movement of water, solutes and cells between body compartments. However this function is highly regulated and specialised in different epitheilia to allow secretion, absorption, protection, transcellular transport, sensation detection, and selective permeability, depending on the specific requirements of the epithelium. Tight cell-cell contacts are vital for this barrier function, and are also important for various other processes that occur during development and during adult life. For example, during morphogenesis, cell shape and motility changes direct the transformation of uniform sheets of cells into specialized three-dimensional structures (Hogan, 1999). While this requires dynamic remodeling of some cell-cell adhesions (see section 1.4 for more detail), some groups of cells must maintain their tight adhesion. Furthermore, during wound healing, groups of cells must move together coordinately to close the wound without disrupting their cell-cell contacts (Gumbiner, 1996; Vasioukhin and Fuchs, 2001). Cell-cell contacts are also of importance in the nervous system and in endothelia (Dejana, 2004; Yamada and Nelson, 2007).

It has long been understood that tight cell-cell adhesions are mediated by specific structures at the interface between cells, known as cell-cell junctions. Electron microscopy allowed the identification of three main structures at the cell-cell contacts of the epithelium, which correspond to three main types of cell-cell junctions mediating cell adhesion (Farquhar and Palade, 1963). These junctions are depicted in Figure 1.2.

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Figure 1.2 ***Cell-cell junctions in epithelial cells.*** *(A) Schematic representation of the three major types of cell-cell adhesion junctions in epithelial cells. Tight junctions are found at the apical side of the cells and link cells together through the binding of claudin and occludin proteins. These form a water-tight barrier between the cells. Cadherin proteins form the core of the Adherens junction. Both tight junctions and adherens junctions are linked to the actin cytoskeleton. Desmosomes are formed by the interaction between desmosomal cadherins and are linked to intermediate filaments. (B) Electron micrograph showing tight junctions, adherens junctions and desmosomes formed between two murine intestinal epithelial cells. Schematic and electron micrograph taken from Perez-Moreno et al., 2003.*

Tight junctions, or zonula occludens, the most apical of the junctional complexes in a polarised epithelial cell, are formed by claudin and occludin proteins (Aijaz et al., 2006). These junctions can act as a seal preventing the passage of water, small molecules and ions through the space between the cells. Moreover, tight junctions block the movement of membrane proteins between the apical and basolateral surfaces of the cell (Shin et al., 2006), which is important for the specialised functions of the two surfaces and for the maintainance of cellular polarity.

Positioned directly basally to the tight junctions are structures known as the adherens junctions, or *zonula adherens* (Figure 1.2). These junctions are held

together by Ca^{2+}-dependent transmembrane molecules known as cadherins (Yap et al., 1997). There is much evidence to suggest that establishment of adherens junctions is a crucial early event in epithelial sheet formation and polarisation (Braga and Yap, 2005; Yeaman et al., 1999). Many studies have shown that the prior formation of adherens junctions is required for the formation of other junctional complexes, and that this may involve cadherin-triggered signalling (Balda et al., 1993; Kohler et al., 2004; Tunggal et al., 2005; van Hengel et al., 1997). Both tight junctions and adherens junctions are linked to the actin cytoskeleton.

Desmosomes are specialised intercellular junctional complexes which are attached to the intermediate filament cytoskeleton (Kowalczyk et al., 1999). Cell-cell adhesion at desmosomes is mediated through the desmosomal cadherins desmocollin and desmoglein. The crucial role of desmosomes in cell-cell adhesion is highlighted by a number of diseases resulting from defective desmosomes (McMillan and Shimizu, 2001). The formation of desmosomal cell-cell junctions stabilises cell-cell contacts initiated by adherens junctions and specifically blocking the formation of desmosome formation in epidermis disrupts adherens junctions (Vasioukhin et al., 2001b). Thus mature adherens junctions may require desmosomes for their stability.

In addition to the junctional complexes described above, gap junctions are found more basolaterally and are more widespread, being found in most animal tissues. Gap junctions constitute a cluster of channels, thus allowing small water-soluble molecules to be passed between adjacent cells. This allows cells to be coupled metabolically (Evans et al., 2006).

Adhesion at cell-cell junctions is mediated by interactions between cell adhesion molecules (CAMs). CAMs mediate cell-cell adhesion through dimerisation with other CAMs in adjacent cells. This may be heterophilic or homophilic dimerisation. In epithelial cells E-cadherin and claudins function as CAMs at adherens junctions and tight junctions, respectively. There are many other CAMs found at cell-cell contacts including a group of immunoglobulin (Ig)-like CAMs, which contain at least one Ig domain. For example, Junctional adhesion

molecules (JAMs) are a group of Ig-like CAMs found in epithelial and endothelial cells which are involved in recruitment of cell polarity proteins (Ohno, 2001). Other members of the Ig-like CAM family can be found in many different cell types and are important in the regulation of a variety of cellular processes including cell adhesion and migration (Brummendorf and Lemmon, 2001). Nectins are a group of Ig-like CAMs which play a number of roles in cell-cell adhesion in epithelia and also at synapses (Irie et al., 2004). Their adhesive activity is through homophilic dimerisation of nectin molecules. Initial nectin-based cell-cell adhesion is important for the formation of adherens junctions and subsequent formation of tight junctions (Irie et al., 2004). Formation of nectin-based cell-cell contact leads to the recruitment of cadherins to the adhesion site through afadin and catenin molecules. Nectins also involved in the recruitment of JAMs, claudins and occludin to cell-cell contacts (Irie et al., 2004). Furthermore, nectin-based cell-cell adhesion is also thought to important in mediating contact inhibition of cell proliferation and cell movement (Fujito et al., 2005).

There is a wealth of evidence implicating the involvement of cell-cell junctions, particularly the adherens junction, in cellular signalling, although the molecular mechanisms are not fully understood (Fagotto and Gumbiner, 1996; Jamora and Fuchs, 2002; Perez-Moreno et al., 2003; Wheelock and Johnson, 2003a). In this thesis a novel pathway for signalling from adherens junctions is investigated.

1.2 The adherens junction

1.2.1 The cadherin superfamily

The transmembrane core of the adherens junction consists of glycoproteins known as classical cadherins which were first identified as calcium-dependent mediators of cell-cell adhesion (Yoshida-Noro et al., 1984). The name cadherin stems from "calcium-dependent cell-cell-adhesion system". A large number of cadherin genes have now been identified which form the cadherin superfamily. This includes classical cadherins, desmosomal cadherins, protocadherins and atypical cadherins (Yagi and Takeichi, 2000). All members of the cadherin superfamily contain multiple tandem copies of the extracellular (EC) repeat within their extracellular domain. EC repeats are involved in the binding of Ca^{2+} ions, which is crucial for the adhesive activity of cadherins (Takeichi, 1990).

E-cadherin, which is expressed mainly in epithelia, is the prototype and best-characterised member of the classical cadherins. Other classical cadherins which have also been well characterised include N-cadherin and VE-cadherin that are mainly expressed in neuronal tissues or in endothelium respectively. However the classical cadherin family is large and there are many other important members (Steinberg and McNutt, 1999; Wheelock and Johnson, 2003b). Different members of the classical cadherin family have distinct properties. For example, increased expression of 'mesenchymal' cadherins (N-cadherin, R-cadherin, and cadherin 11) increases the motility and invasiveness of epithelial cells (Feltes et al., 2002; Hazan et al., 2000; Nieman et al., 1999b). Desmosomal cadherins are crucial adhesive proteins in the desmosomes as described in section 1.1. Protocadherins typically contain 6 or 7 EC repeats in their extracellular domain regions, but protocadherins with as many as 11 or as few as 4 EC repeats have been identified. Their intracellular regions are highly variable compared to classical cadherins. Although there is evidence that they can mediate cell-cell adhesion, it is considered to be much weaker than that mediated by the classical cadherins (Frank and Kemler, 2002; Suzuki, 1996).

1.2.2 Components of the adherens junction

As single-pass transmembrane proteins, classical cadherins mediate cell-cell adhesion at the adherens junction via Ca^{2+}-dependent homophilic interactions with other cadherins expressed in adjacent cells (Figure 1.3). These *trans* dimers with cadherins in adjacent cells also form *cis* dimers with cadherins in the same cell membrane. This enables cadherin dimers to cluster to form the mature adherens junction, a process known as the 'zipper' model (Figure 1.3) (Gumbiner, 1996). The intracellular domain of classical cadherins interacts with members of a family of armadillo-repeat-containing proteins known as catenins (Perez-Moreno and Fuchs, 2006). Interactions with catenins occur via two Cadherin Homology (CH) domains: CH2 domain (located at the membrane proximal region) and CH3 domain (located at the distal region). These domains are conserved between classical cadherins. The CH2 and CH3 domains of classical cadherins interact with p120-catenin (p120) and β-catenin, respectively (McCrea et al., 1991; Reynolds et al., 1994) (Figure 1.3). Plakoglobin is highly homologous to β-catenin and usually associates with desmosomal cadherins, but can also act in an analogous way to β-catenin by associating with classical cadherins (Knudsen and Wheelock, 1992). Furthermore, E-cadherin specifically binds other proteins. The CH2 domain of E-cadherin also interacts with Hakai, an E3 ubiquitin ligase which regulates the stability of E-cadherin-based cell-cell contacts (discussed further in section 1.4.4) (Fujita et al., 2002; Tricaud et al., 2005). C3G, a guanine nucleotide exchange factor for the small GTPase Rap1, also interacts transiently with the CH3 domain of E-cadherin and is important for the establishment of cell-cell contacts (Hogan et al., 2004).

Figure 1.3 ***Core components of the adherens junction and a current model for how α-catenin may promote bundling of actin filaments at sites of cell-cell contacts.*** *Classical cadherins contain five extracellular (EC) repeat domains which mediate calcium-dependent homophilic interactions between cadherin molecules from neighbouring cells. Cadherin trans dimers also dimerise in cis which leads to the formation of cadherin clusters, thus forming the core of the adherens junction. Through the juxtamembrane CH2 domain (shown in pink), cadherins bind to p120. Through the membrane distal CH3 domain (shown in blue), cadherins bind to β-catenin (β) which links them to α-catenin (α). α-catenin is known to link the cadherin/catenin complex to actin bundles. The mechanism by which α-catenin can promote the association of the actin cytoskeleton to adherens junctions is being actively researched but a current model suggests that α-catenin binds to β-catenin as a monomer, but can dissociate from β-catenin and dimerise. Dimeric α-catenin is proposed to inhibit Arp2/3-mediated actin filament branching and therefore promote the formation of bundled actin filaments. Schematic adapted from Weis and Nelson, 2006.*

1.2.3 Roles of β-catenin and α-catenin at the adherens junction in linking to the actin cytoskeleton

β-catenin binds to another catenin protein known as α-catenin. α-catenin can bind directly to filamentous actin (Pokutta et al., 2002; Rimm et al., 1995) and also binds to several actin-binding proteins (Kobielak and Fuchs, 2004). α-catenin plays a crucial role in the remodeling of the actin cytoskeleton into radial actin cables and genetic studies in mice have shown α-catenin is required for sustained adhesion between cells during morphogenesis (Adams et al., 1998; Torres et al., 1997; Vasioukhin et al., 2001a). It has long been considered that α-catenin's interaction with both the classical cadherin complex and actin provides the link between the adherens junction and the actin cytoskeleton which is vital for its adhesive function. However a series of recent experiments have raised doubt over the the molecular basis of the link from the adherens junction to the actin cytoskeleton (Drees et al., 2005; Weis and Nelson, 2006; Yamada et al., 2005).

Some cell lines lacking endogenous α-catenin can form cell adhesions, albeit less well than those 'rescued' with ectopic α-catenin (Imamura et al., 1999; Watabe-Uchida et al., 1998). Direct binding studies using purified proteins and measurement of protein dynamics in live cells have indicated that the binding of α-catenin to the E-cadherin/β-catenin complex complex and actin are mutually exclusive (Drees et al., 2005; Weis and Nelson, 2006; Yamada et al., 2005). When E-cadherin, α-catenin and β-catenin purified receombinant proteins were mixed, a fraction of α-catenin, but not E-cadherin or β-catenin bound to actin. α-catenin can form homodimers which compete with β-catenin for α-catenin binding (Yamada et al., 2005). To ensure these results were not simply due to the formation of α-catenin dimers preventing the interaction of α-catenin with β-catenin, a chimera comprising the α-catenin binding site of β-catenin fused to α-catenin was produced. This chimera did not interact with actin, suggesting that α-catenin cannot bind to actin and the E-cadherin/β-catenin complex simultaneously (Drees et al., 2005). Futhermore, fluorescence recovery after photobleaching measurements in live cells showed that E-cadherin, β-catenin

and α-catenin had almost identical recovery times but those of actin were much faster (Yamada et al., 2005). If a stable complex existed between the E-cadherin/catenin complex and actin, it would be expected that diffusion properties would be more similar.

Further experiments are required to fully understand the links between the E-cadherin/catenin complex and actin, but a model has been proposed (Figure 1.3) (Weis and Nelson, 2006). This model suggests that a local concentration of α-catenin monomers is produced in proximity to the E-cadherin/β-catenin clusters, via its binding to β-catenin. α-catenin can interchange between its monomeric state (where it is bound to β-catenin) and its dimeric state in the cytosol. Dimeric α-catenin can potently inhibit the Arp2/3 complex which promotes the branching of actin filaments (Figure 1.3) (Drees et al., 2005). Hence, dimeric α-catenin could promote the bundling of actin filaments at the adherens junction. It is also possible that other proteins may enable the anchoring of actin filaments at the adherens junction. For example nectins are found at adherens junctions and can bind l-afadin, which binds to both actin and α-catenin (Irie et al., 2004).

1.2.4 Roles of p120 at the adherens junction

p120 was initially identified as a src substrate and has since been characterised as the prototype and most abundant member of the subfamily of p120 armadillo related proteins, which includes δ-catenin, ARVCF and p0071 (Reynolds, 2007; Reynolds et al., 1994; Reynolds et al., 1992). There are many splice variants of p120, but typically in mouse and humans, four main isoforms are expressed (Keirsebilck et al., 1998). All of the four main human isoforms contain the central Armadillo (Arm) repeat region, which contains 10 Arm repeats and is required for cadherin binding (Daniel and Reynolds, 1995). Arm repeats represent a protein-protein interaction domain and are sequences of approximately 45 amino acids which are tandemly repeated. They are found in β-catenin and plakoglobin, as well as many other proteins of diverse cellular functions (Peifer et al., 1994). p120 is now known to have various roles in the regulation of cell adhesion via dynamic regulation of the actin cytoskeleton,

transport of cadherins to the cell membrane and stability of cadherins at the cell membrane (Anastasiadis and Reynolds, 2001; Perez-Moreno and Fuchs, 2006; Reynolds and Carnahan, 2004).

p120 has emerged as a key regulator of cadherin turnover, modulating both transport and stability of cadherins at the plasma membrane (Xiao et al., 2007). A mutated form of E-cadherin which is selectively uncoupled from p120 but not other catenins, was shown to disrupt cell-cell adhesion (Thoreson et al., 2000). Cadherin-deficient cells were transfected with either wild type or p120-uncoupled E-cadherin. Those transfected with wild type E-cadherin formed tight cell-cell contacts with a continuous border of actin filaments at the junctions, whereas those transfected with p120-uncoupled E-cadherin formed weaker contacts and showed a disorganised actin cytoskeleton (Thoreson et al., 2000). Furthermore cell aggregates formed by cells expressing p120-uncoupled E-cadherin were dissociated by pipetting, compared to wild type E-cadherin expressing cells where aggregates remained upon pipetting (Thoreson et al., 2000). By studying cell types with very low expression levels of p120, or where where p120 expression has been knocked down, it has been shown that a lack of p120 leads to an increase in both the internalisation and degradation of cadherins (Davis et al., 2003; Ireton et al., 2002; Maeda et al., 2006; Xiao et al., 2003; Xiao et al., 2005). Cadherins are also destabilised by targeted p120 ablation in mice (Davis and Reynolds, 2006). There are a number of possible mechanisms by which p120 could function to regulate cadherin turnover. It has been suggested that p120 could act as a 'cap' by shielding sequences in the E-cadherin tail that interact with clathrin adaptor proteins (Xiao et al., 2005; Xiao et al., 2007). Furthermore, p120 competes with two other proteins for the binding to E-cadherin, Hakai and γ-secretase. Hakai leads to the downregulation of E-cadherin after phosphorylation by tyrosine kinases and is discussed further in section 1.4.4 (Fujita et al., 2002; Tricaud et al., 2005). γ-secretase binds to and cleaves E-cadherin, resulting in the disassembly of adherens junctions, and is discussed further in sections 1.4.5 and 1.5 (Marambaud et al., 2002; Wu et al., 2007). Thus by competing with Hakai and γ-secretase for binding to E-cadherin, p120 could

protect E-cadherin from internalisation or degradation. Moreover, p120 also interacts with microtubules (Franz and Ridley, 2004), and some studies suggest that cadherin transport to the plasma membrane along microtubule tracks can be enhanced by the interaction between p120 and kinesin motor proteins (Chen et al., 2003; Yanagisawa et al., 2004).

Surprisingly, despite this abundance of data showing that p120 plays a positive role in the stability of cadherins at the plasma membrane, some reports have suggested that p120 could act to destablise the adhesive activity of cadherins in certain cell types (Aono et al., 1999; Ohkubo and Ozawa, 1999; Ozawa and Kemler, 1998). For example, in Colo205 cells which are largely non-adherent even though they possess essentially intact E-cadherin-catenin complexes, expression of an N-terminally deleted p120 construct restored adhesiveness (Aono et al., 1999). This data could be explained by an aberrant signalling pathway in Colo205 cells which could inactivate p120's ability to promote adhesion. However it is supported by other studies using E-cadherin constructs that were truncated or mutated in the p120-binding regions, suggesting an inhibitory role of p120 in adhesion in K-562 leukemia cells and L-fibroblasts (Ohkubo and Ozawa, 1999; Ozawa and Kemler, 1998). In all of these studies, inhibition of adhesion correlated with hyperphosphorylation of p120, suggesting that phosphorylation of p120 could affect its role in cadherin stability and turnover. Further work is required to fully understand these apparent contradictions in the role of p120. It is possible that p120 in general has a positive role in cadherin stability at the plasma membrane, but when certain signalling pathways are activated, possibly resulting in p120 phosphorylation, it may also have a negative role (Anastasiadis and Reynolds, 2000).

p120 is highly phosphorylated at multiple tyrosine, serine and threonine residues (Alema and Salvatore, 2007). Thus, as a protein with varied and crucial roles at the adherens junction, it is widely thought that p120 phosphorylation is likely to be important in the fine tuning of its activities. However despite the large number of phosphorylation sites present, the role of p120 phosphorylation remains incompletely understood. Indeed, although p120 was first identified as a src substrate, the role of src phosphorylation and its possible link to cellular

transfomation is still not clear (Reynolds, 2007). However, as described further in section 1.3.5, a role for src phosphorylation in p120 signalling has recently been described (Hosking et al., 2007). Studies into the effect of tyrosine phosphorylation on its association with cadherin have produced apparently contrasting results and may reflect a fine and complex regulation of this association (Reviewed in Alema and Salvatore, 2006). p120 also associates with the tyrosine kinases Fer, Yes and Fyn as well as several tyrosine phosphatases, suggesting that p120 could act as a scaffold for the recruitment of tyrosine phosphorylation modulators, pointing to a role of p120 in the integration of cellular signals (Reynolds and Roczniak-Ferguson, 2004). The role of serine/threonine phosphorylation of p120 is also still largely unclear. However serine/threonine phosphorylation of p120 can be induced by its membrane localisation and can influence E-cadherin dynamics at the plasma membrane (Fukumoto et al., 2007; Xia et al., 2006). The recent generation of phosphospecific p120 antibodies is likely to accelerate the accumulation of knowledge into the role of p120 phosphorylation (Xia et al., 2004).

As will be discussed further in section 1.3.2, p120 plays a crucial role in signalling to the actin cytoskelton through the regulation of Rho GTPases. Additionally, both β-catenin and p120 can be localised in the nucleus of cells and have roles in the transmission of cellular signals to the nucleus (further described in sections 1.3.4 and 1.3.5).

1.3 Signalling mediated by cadherins.

1.3.1 Functional roles of cadherin-mediated signalling

A large body of evidence has accumulated implicating cadherins in the induction of intracellular signals (Knudsen et al., 1998; Perez-Moreno et al., 2003; Wheelock and Johnson, 2003a). These signalling pathways include the regulation of cell proliferation (Levenberg et al., 1999; Sasaki et al., 2000; St Croix et al., 1998), cell survival (Fouquet et al., 2004; Kang et al., 2007; Vallorosi et al., 2000), and differentiation (Goichberg and Geiger, 1998; Larue et al., 1996). Hence, in addition to their structural and adhesive roles, cadherins also appear to

play a vital role in transducing signals particularly during development (Halbleib and Nelson, 2006; Perez-Moreno et al., 2003).

Cadherin-mediated cell-cell adhesion has long been known to suppress cell proliferation, a process known as contact inhibition of growth (Hermiston and Gordon, 1995b; Navarro et al., 1991; Takahashi and Suzuki, 1996; Watabe et al., 1994). Both E-cadherin and N-cadherin have been shown to upregulate the cyclin-dependent kinase (CDK) inhibitory protein p27 (Levenberg et al., 1999; St Croix et al., 1998). The activity of CDKs is required for progression through the cell cycle. The tight regulation of CDKs (mediated through binding of cyclins or inhibitory subunits such as p27 and also by phosphorylation) is crucial for the control of cell proliferation (Morgan, 1995). In a study using cells lacking E-cadherin, introduction of E-cadherin expression resulted in reduced proliferation, accompanied by an increase in p27 levels (St Croix et al., 1998). Additionally, E-cadherin-neutralising antibodies stimulated proliferation of cells endogenously expressing E-cadherin, and reduced p27 levels. Crucially, forced expression of p27 blocked the stimulation of proliferation induced by cadherin-neutralising antibodies, suggesting that p27 upregulation is at least partly responsible for E-cadherin-mediated proliferation suppression (St Croix et al., 1998).

Several studies have also indicated a role for E-cadherin in the suppression of apoptosis, particularly in protecting cells from detachment-induced apoptosis, known as anoikis (Fouquet et al., 2004; Kang et al., 2007; Vallorosi et al., 2000). Normal nonhematopoietic cells typically undergo anoikis in the absence of attachment to the ECM (Frisch and Screaton, 2001). This is thought to prevent ectopic growth at inappropriate body sites, as anchorage-independent growth is a defining property of transformed cells (Schwartz, 1997). Several reports have indicated that formation of cadherin-based cell-cell adhesions suppresses anoikis of epithelial cells under matrix-deficient conditions (Hermiston and Gordon, 1995a; Peluso et al., 1996; Rak et al., 1999; Tran et al., 2002). A study of anoikis in enterocytes showed that upon loss of anchorage, these cells undergo rapid apoptosis which involves early loss of E-cadherin executed by lysosomal- and proteasomal-dependent degradation (Fouquet et al., 2004). Activation of E-cadherin using an E-cadherin-Fc chimera protein, reduces this anoikis, whereas

treatment with blocking antibodies to E-cadherin increases it (Fouquet et al., 2004).

The importance of cadherins in development is shown by abnormal tissue maturation and embryonic lethality of mice null for specific cadherins (Halbleib and Nelson, 2006; Hynes, 1996). There is now good evidence that the role of cadherins in development extends beyond their structural role and that through induction of signalling, they have a morphoregulatory role (Knudsen et al., 1998). A direct involvement of N-cadherin signalling in muscle differentiation has been indicated by a number of studies, either by activating N-cadherin with recombinant N-cadherin ligands, or by blocking N-cadherin with antibodies, or with dominant-negative mutants. These studies have linked N-cadherin-mediated cell-cell adhesion to upregulation of two myogenic regulatory factor proteins, MyoD and myogenin (Goichberg and Geiger, 1998; Holt et al., 1994; Imanaka-Yoshida et al., 1998; Linask et al., 1997). Other cadherins such as E-cadherin, R-cadherin and M-cadherin can also impact on muscle differentiation (Redfield et al., 1997; Rosenberg et al., 1997; Zeschnigk et al., 1995). Muscle is not the only cell type whose differentiation has been shown to be affected by cadherins. Similar studies have shown effects on thymocyte and erythroid differentiation and mammary gland development (Armeanu et al., 1995; Muller et al., 1997; Radice et al., 1997). A study by Larue et al. in 1996 showed for the first time that specific cadherin types can stimulate specific signals leading to the differentiation of different types of tissues. Null mutant mouse embryonic stem (ES) cells for E-cadherin were used, which showed an aggregation defect and a change in the expression levels of the transcription factor T-brachyury. Although aggregation could be restored by overexpression of either E- or N-cadherin, there were differences in gene expression and tissue formation depending on which cadherin was used to 'rescue' the cells. Overexpression of E-cadherin but not N-cadherin could rescue the wild-type regulation of the expression of T-brachyury (Larue et al., 1996). This indicated that specific cadherins can link information from the cell surface to gene expression in the nucleus. Additionally, when the ES cells were injected subcutaneously into syngenic hosts to form teratomas, the E-cadherin-rescued ES cells resulted exclusively in epithelial structures, but N-

cadherin-rescued ES cells did not have epithelia but instead developed neuroepithelia and cartilage (Larue et al., 1996).

It is clear that cadherins have multiple signalling roles and links have been shown to exist between cadherins and regulation of the levels of signalling proteins such as p27, transcription factors and myogenic regulators. However the molecular mechanisms by which cadherins induce signals in order to regulate cellular behaviour and modulation of transcription remain incompletely understood. The following sections describe what is known about signalling mechanisms induced by cadherins and their binding proteins.

1.3.2 Cadherin-mediatated signalling through Rho GTPases

Rho GTPases are a family of small GTPases that act as molecular switches and control a wide variety of cell signalling events (Etienne-Manneville and Hall, 2002). Briefly, Rho GTPases cycle between an active GTP-bound state and an inactive GDP-bound state. They can be activated by specific gunanine nucleotide exchange factors (GEFs) or inactivated by GTPase activating proteins (GAPs). Rho GTPases are most well known for their role in the control of the actin cytoskeleton. RhoA, rac1 and cdc42 promote the formation of actin-rich stress fibres, lamellipodia and filopodia, respectively. Lamellipodia and filopodia allow the formation of actin-based protrusions and extensions which are important for cell motility (Etienne-Manneville and Hall, 2002). As described in section 1.2.3, bundled actin filaments are linked to adherens junctions and are vital for their adhesive function. Since cadherins are dynamically regulated during morphogenesis (described in more detail in section 1.4), their ability to modulate their associated actin cytoskeleton is also crucial. The relationship between cadherins and Rho GTPase activity is complex and involves both inside-out signalling with Rho GTPase activity modulating cadherin function, and outside-in signalling with cadherins signalling to Rho GTPases.

That Rho GTPases promote the establishment and maintainance of cadherin-based cell-cell contacts was first demonstrated in a study using dominant negative Rac, or C3 toxin to block endogenous Rho activity in keratinocytes

(Braga et al., 1997). Cells were cultured in low calcium medium which prevents cadherin-based cell-cell adhesion, as calcium is required for dimerisation of the cadherin extracellular domain. Restoring calcium levels with normal medium promotes formation of cell-cell contacts in normal keratinocytes, but is blocked in the presence of C3 toxin or dominant negative Rac (Braga et al., 1997). This study also showed that Rho and Rac are required for the accumulation of actin at the sites of cell-cell contact (Braga et al., 1997). One mechanism for inside-out signalling from Rho GTPases to cadherins is through the target of Rac1 and Cdc42, IQGAP1. IQGAP1 competes with α-catenin for binding to β-catenin, reducing adhesion strength. Active GTP-bound forms of Rac1 and Cdc42 bind to IQGAP1, which inhibits the interaction between IQGAP1 and β-catenin, thus strengthening the cadherin/catenin complex (Fukata et al., 1999; Kuroda et al., 1998). Another small GTPase from the Ras family, Rap1, has been shown to positively regulate the formation of E-cadherin based cell-cell contacts in epithelial cell lines (Hogan et al., 2004; Price et al., 2004). Through recruitment of C3G, a GEF for Rap1, to the E-cadherin intracellular domain, Rap1 is activated at sites of nascent cell-cell contacts and is required for proper targeting of E-cadherin to the membrane (Hogan et al., 2004). Interestingly, Rap1 was shown to function upstream of Cdc42 in this process.

There is evidence suggesting that cadherin-mediated cell-cell contacts leads to the activation of Rac1 and Cdc42 wheras RhoA activity decreases as cells become more confluent, although this may depend on the experimental system used (Noren et al., 2001). Several studies by numerous laboratories using different techniques have shown that Rac1 is activated at sites of initial cell-cell contact, and some of these studies have implicated a dependence on either phosphatidylinositol-3-kinase (PI3K) or receptor tyrosine kinases (RTKs) in the cadherin-mediated recruitment of Rac1, depending on the cell type (Betson et al., 2002; Ehrlich et al., 2002; Kovacs et al., 2002). A recent study suggested that cadherin ligation leads to Rac1 activation through upregulation of Vav2, a GEF for Rac1, via a pathway dependent on Src, Rap1 and PI3K (Fukuyama et al., 2006). Interestingly, uncoupling the interaction between cadherins and p120

prevents Rac1 activation at cell-cell contacts (Goodwin et al., 2003). Indeed, p120 may be an important connection between cadherins and Rho GTPases.

Overexpression of p120 in NIH3T3 fibroblast cells leads to an unusual 'branching' phenotype with the production of long processes from the cells (Reynolds et al., 1996). p120 has been proposed to bind to and prevent RhoA activity by stabilising the GDP-bound form of Rho, which is involved in the p120-induced branching phenotype (Anastasiadis et al., 2000; Magie et al., 2002). However p120 can also activate Rac1 and Cdc42 most likely through its interaction with Vav2 (Anastasiadis and Reynolds, 2000; Grosheva et al., 2001; Noren et al., 2000). There are some discrepancies in the literature about whether p120 must be free in the cytoplasm to affect the activity of Rho GTPase activity, or whether it can regulate them as a part of the adherens junction complex. The study described above by Goodwin et al. in 2003 suggests that p120 binding to E-cadherin is required for the activation of Rac1. However other studies have shown that sequestering p120 at adherens junctions by expressing E-cadherin abolishes its effects on cell morphology (Grosheva et al., 2001). The idea that p120 must be free in the cytoplasm to modulate Rho GTPase activity led to a model suggesting that at high cell density and when adherens junctions are stable, p120 is largely associated with cadherin and can not greatly influence actin dynamics. p120 may be released into the cytosol when cell density is reduced (for example during wounding), which may lead to the inhibition of RhoA and activation of Rac1 and Cdc42 resulting in an increase in migration (Grosheva et al., 2001; Noren et al., 2000). However a recent study suggests that mesenchymal cadherins may require the binding of p120 in order to promote a more migratory phenotype (Yanagisawa and Anastasiadis, 2006). Therefore this model may be more complex and depend on the specific cadherin-type expressed in cells (Anastasiadis and Reynolds, 2001). The situation is further complicated by the varied effects of Rho GTPases on cell motility. Although their activities are in general required for cell motility, Rac1 and Cdc42 activation may decrease motility in epithelial cells by increasing cell-cell adhesion. The action of cadherins, p120 and RhoGTPases in regulating cell motility vs cell-cell adhesion may vary depending on their localisation, abundance or activity level as well as the cell type.

A recent paper from the Reynolds laboratory identified an interesting mechanism which may link p120's roles in modulation of RhoGTPases and cadherin turnover (Wildenberg et al., 2006). Rho activity is known to be negatively regulated by Rac, through activation of p190RhoGAP. This study suggested a crucial role for p120 in Rho inhibition by Rac. Platelet derived growth factor (PDGF) receptor-induced Rac activity was shown to induce translocation of p190RhoGAP to the membrane where it can bind adhesion complexes by interacting with p120 (Wildenberg et al., 2006). Knockdown of p120 prevented Rac from inhibiting Rho. Interestingly, the formation of adherens junctions induced by Rac activation was blocked in the absence of p190RhoGAP (Wildenberg et al., 2006). Thus through the p120-p190RhoGAP interaction, Rac may control both adherens junction assembly and Rho inhibition.

1.3.3 Cadherin-mediated signalling through receptor tyrosine kinases

A number of reports have suggested functional interactions between cadherins and receptor tyrosine kinases (RTKs). Most of these studies indicate that cadherins can modulate the activity of tyrosine kinase signalling (Wheelock and Johnson, 2003a). The link between N-cadherin and the fibroblast growth factor (FGF) receptor is the most well characterised. N-cadherin has been suggested to facilitate the dimerisation of the FGF receptor and initiate signalling in the absense of growth factor (Doherty et al., 2000; Skaper et al., 2001). In these studies, the FGF-dependent neurite outgrowth from cultured neurons was assayed. Plating neurons on N-cadherin substrates leads to induction of neurite outgrowth in the absence of FGF, but required signalling downstream of the FGF receptor (Doherty et al., 2000; Skaper et al., 2001). Additionally, another report showed that inhibitors of FGF-receptor signalling reduced N-cadherin-mediated invasion, further implicating a role for N-cadherin in activation of the FGF receptor signalling and the involvement of FGFR signalling in N-cadherin function (Nieman et al., 1999b). Furthermore, N-cadherin has also been shown to promote ligand-dependent FGF receptor signalling by forming a complex with the FGF receptor and stabilising it at the cell surface (Suyama et al., 2002).

Additionally, E-cadherin has been shown to associate with the epidermal growth factor (EGF) receptor (Hoschuetzky et al., 1994). Cell-cell contact has been shown to either inhibit EGF-dependent activation of the EGF receptor (Qian et al., 2004; Takahashi and Suzuki, 1996) or transiently induce ligand-independent activation of the EGF receptor, resulting in the activation of MAP kinases (Pece and Gutkind, 2000). This suggests that there could be variation in the links between E-cadherin and the EGF receptor in different cell types. A recent study used recombinant E-cadherin protein to selectively induce E-cadherin ligation in isolated cells, to determine whether E-cadherin ligation alone could affect EGF receptor signalling, without the complex process of cell-cell contact formation (Perrais et al., 2007). Selective homophilic ligation was shown to inhibit cell proliferation and EGF receptor signalling, but not through autophosphorylation of the receptor and there was no effect on MAP kinases. Instead, a different pathway involving Src and signal transducers and activators of transcription (STAT) 5 was inhibited (Perrais et al., 2007). Furthermore, cell-cell contact formation has also been reported to recruit PI3K to E-cadherin with a concommitant upregulation in phosphorylated tyrosine residues in PI3K (Pece et al., 1999). As discussed in section 1.3.2, activation of tyrosine kinases or PI3K may be involved in the activation of Rac1 at sites of cell-cell contacts. Moreover, a recent report suggest that PI3K and Akt activation by E-cadherin mediated cell-cell adhesion may play a role in suppression of anoikis in Ewing tumour cells (Kang et al., 2007).

1.3.4 The Wnt signalling pathway

In addition to its role in adhesion, β-catenin has a crucial role in the Wnt signalling pathway (Brembeck et al., 2006; Willert and Jones, 2006). Wnts are secreted lipid-modified proteins and a large body of evidence has shown that Wnts are powerful regulators of cell proliferation and differentiation, particularly during embryogenesis (Willert et al., 2003). When cells are stimulated with Wnt, β-catenin is translocated into the nucleus where it binds a transcription factor, T-cell factor/lymphocyte enhancer factor-1 (TCF/LEF-1), and acts as a transcriptional activator (Behrens et al., 1996; Molenaar et al., 1996).

Several loss of function mutations of Wnt generated in the mouse showed severe and varied developmental phenotypes (Parr and McMahon, 1995; Stark et al., 1994; Yang and Niswander, 1995). Wnt signalling has emerged as a conserved mechanism in metazoan animals and controls a variety of processes such as cell fate, cell proliferation vs differentiation, cell survival, cell behaviour, and migration during morphogenesis (Cadigan and Liu, 2006; Moon et al., 2002; Nelson and Nusse, 2004). It is now known that there are numerous signalling pathways downstream of Wnt, but this section focuses on the pathway mediated by β-catenin nuclear translocation, known as the canonical Wnt signalling pathway.

The main components of the canonical Wnt signalling pathway were identified by genetic studies in flies where mutants were identified with phenotypes consistent with defective Wnt signalling. Genetic studies allowed the ordering of these components into a signalling pathway (Figure 1.4), and biochemical studies of the vertebrate counterparts have allowed a good understanding of the interactions between them (Cadigan and Nusse, 1997). Briefly, in the absence of Wnt signalling, β-catenin that is not bound by cadherins at the adherens junction is targeted for ubiquitination and degradation in the proteasome, following priming phosphorylations by the serine/threonine kinases Casein Kinase I (CK1) and glycogen synthase kinase-3β (GSK-3β) (Polakis, 2002). GSK-3β is found in a scaffolding complex with axin and the adenomatous polyposis coli (APC) protein, which are also required for the degradation of β-catenin (Behrens et al., 1998). Wnt binding to its receptor at the cell surface Frizzled, and the co-receptor Lipoprotein receptor related proteins 5 and 6 (LRP-5/6), activates dishevelled which can interact with the Axin/APC/GSK-3β complex (Cliffe et al., 2003). This blocks the Axin/APC/GSK-3β complex from phosphorylating β-catenin. Thus β-catenin is stabilized and accumulates in the cytoplasm, resulting in its nuclear localisation and induction of transcription via its association with TCF/LEF-1 (Behrens et al., 1996).

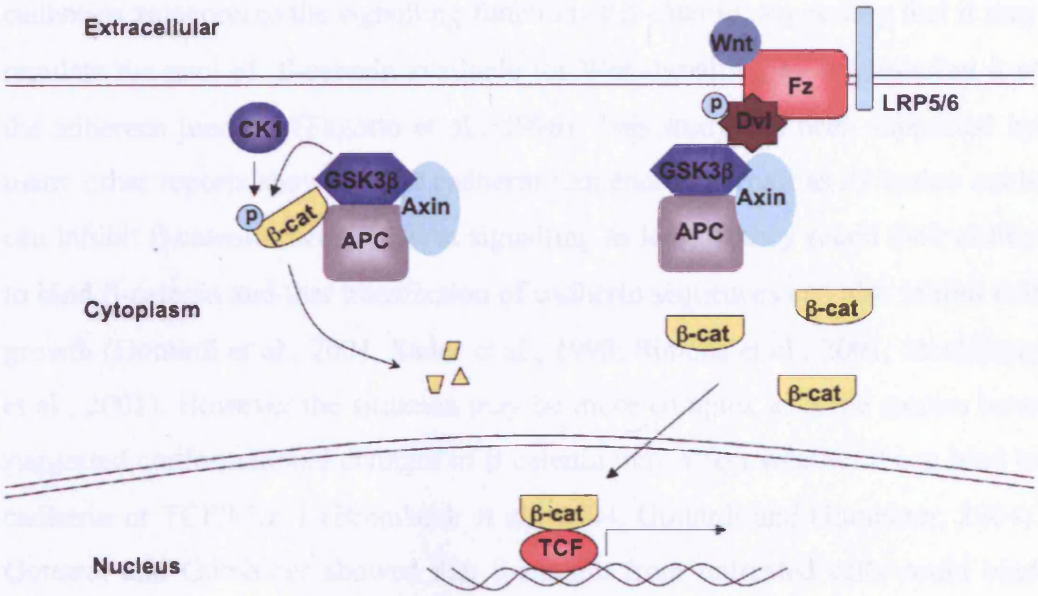


Figure 1.4 ***The canonical Wnt signalling pathway*** *In the absence of a Wnt signal, β-catenin that is not bound to cadherins is targeted for ubiquitination and degradation in the proteasome. Phosphorylations by CK1 and GSK-3β are required to signal the ubiquitination of β-catenin. GSK-3β is found in a complex with APC and axin. Wnt signalling through binding to Frizzled (Fz) and LRP5/6 activates dishevelled (dvl) which blocks the activity of the Axin/APC/GSK-3β complex. Thus β-catenin is stabilized and can enter the nucleus where it can activate transcription through its interaction with TCF/LEF-1.*

Target genes of the canonical Wnt signalling pathway are varied and include many developmental regulators and also genes controlling cell proliferation, cell-cell interactions and cell-matrix interactions (Hecht and Kemler, 2000; Miller et al., 1999). In various cancers, Wnt target genes are also upregulated, including matrix metalloproteases, c-myc and cyclin D1 (Crawford et al., 1999; He et al., 1998; Tetsu and McCormick, 1999). Indeed mutations in Wnt signalling components have been identified in several cancers. In particular the APC gene is altered in individuals with adenomatous familial polyposis as well as in some sporadic colon cancers, where impaired function of APC results in elevated β-catenin levels (Moon et al., 2002; Munemitsu et al., 1995).

β-catenin binds to cadherins and TCF/LEF-1 via the same region, and its binding to cadherins and TCF/LEF-1 has been shown to be mutually exclusive (Graham et al., 2000). Thus it has long been speculated that cadherins could negatively

regulate β-catenin-mediated Wnt signalling pathways. Indeed transfection of cadherins antagonizes the signalling function of β-catenin, suggesting that it may regulate the pool of β-catenin available for Wnt signalling by sequestering it at the adherens junction (Fagotto et al., 1996). This study has been supported by many other reports showing that cadherin sequences as small as 23 amino acids can inhibit β-catenin-mediated Wnt signalling as long as they retain their ability to bind β-catenin and that transfection of cadherin sequences can also inhibit cell growth (Gottardi et al., 2001; Sadot et al., 1998; Simcha et al., 2001; Stockinger et al., 2001). However the situation may be more complex as some studies have suggested conformational changes of β-catenin may affect whether it can bind to cadherin or TCF/LEF-1 (Brembeck et al., 2004; Gottardi and Gumbiner, 2004). Gottardi and Gumbiner showed that β-catenin from untreated cells could bind both TCF/LEF-1 and cadherin equally well, but that β-catenin from Wnt treated cells showed preferential binding to TCF/LEF-1, which they suggested was due to a conformational change within the Arm repeats. Therefore it may be that other factors are involved in the balance between β-catenin's function in Wnt signalling and at adherens junctions.

1.3.5 The roles of p120 in signalling to the nucleus

Nucleocytoplasmic shuttling of p120

p120 is found at several cellular locations including the adherens junction and microtubules. Additionally, under conditions where cadherins are downregulated, p120 has been shown to be found in the cytosol (Roczniak-Ferguson and Reynolds, 2003; van Hengel et al., 1999). In contrast to α- and β-catenins, p120 has been shown to be metabolically stable in cadherin-deficient cells and is found in high levels in the cytosol (Thoreson et al., 2000). Furthermore, in some cancers a cytoplasmic localisation of p120 accompanied by a loss of cadherins, has been reported (Reynolds and Carnahan, 2004; Sarrio et al., 2004). A possible role for cytoplasmic p120 in enhancing metastasis is being actively studied and may be related to its ability to enhance motility by regulating Rho GTPase activity (Anastasiadis and Reynolds, 2000; Reynolds and

Carnahan, 2004). It is also possible that p120 may participate in other signalling pathways in the cytoplasm or nucleus.

Nuclear localisation of p120 has been reported in several cell lines and human tumours (Lo Muzio et al., 2002; Roczniak-Ferguson and Reynolds, 2003; van Hengel et al., 1999). However, in contrast to β-catenin, the nuclear localisation of p120 is not generally observed in most cell lines and treatment of cells with leptomycin B, an inhibitor of nuclear export, is often required to observe nuclear accumulation (Kelly et al., 2004; Roczniak-Ferguson and Reynolds, 2003; van Hengel et al., 1999). This suggested that there is rapid nucleocytoplasmic shuttling of p120 which may be subject to tight regulation.

As shown in Figure 1.5, p120 contains both nuclear localisation sequences (NLS) and nuclear export sequences (NES).

Figure 1.5 ***Domain structure of p120****. p120 contains an N-terminal coiled-coil domain, and 10 Arm repeat domains. An NLS sequence is found between the sixth and seventh Arm repeats. Two NES sequences are also found. There are also many sites of phosphorylation in p120 (denoted by asterisks). Schematic adapted from Daniel et al., 2007.*

NLS sequences bind to the importin complex which mediates the nuclear localisation of proteins. The importin complex consists of Importin-α, which binds directly to the NLS sequence of proteins, and Importin-β which mediates translocation of proteins through the nuclear pore complex (Lange et al., 2007). There is some conflict in the literature about whether the NLS sequence in p120 (located between the sixth and seventh Arm repeats, Figure 1.5) is responsible for nuclear localisation of p120. One study showed that the NLS sequence mediated the nuclear localisation of p120 (Kelly et al., 2004), whereas another study showed that deleting the NLS had no effect on p120's nuclear localisation,

but instead suggested that nuclear localisation is mediated by Arm repeats 3 and 5 (Roczniak-Ferguson and Reynolds, 2003). The discrepancy may be because in the Roczniak-Ferguson study, the NLS was deleted rather than mutated which could affect protein folding, however it raises the interesting possibility that p120's nuclear localisation could be intrinsically conferred by its Arm domain. Importin-α contains an Arm domain which is the solely functional domain within this protein (Conti et al., 1998). An intriguing observation is that β-catenin binds directly to Importin-β and is imported into the nucleus in the absence of Importin-α (Fagotto et al., 1998; Yokoya et al., 1999). Other Armadillo-domain-containing proteins have been shown to be localised in the nucleus, suggesting that the Arm domain may be sufficient for nuclear localisation, raising the possibility that p120 and other Arm-domain containing proteins could function as nuclear transport proteins (Daniel, 2007).

At present the signals triggering nuclear localisation and/or export of p120 are incompletely understood. Glis2, a transcription factor which binds p120, has been shown to induce substantial nuclear localisation of p120 (Hosking et al., 2007) and overexpression of a transmembrane adhesion protein Mucin-1 correlates with p120 nuclear localisation (Li and Kufe, 2001). It will be interesting to determine whether cadherin engagement or cell-cell contact formation has an impact on p120 nuclear localisation.

p120 binds to and regulates the acivity of two transcription factors

A transcriptional repressor Kaiso was identified several years ago as a p120 binding partner in a yeast 2-hybrid screen (Daniel and Reynolds, 1999). Kaiso is a member of the BTB/POZ (Broad complex, Tramtrak, Bric a brac/Pox virus and zinc finger) subfamily of zinc-finger-containing transcription factors. It contains an N-terminal POZ domain which facilitates Kaiso homodimerisation as well as heterodimersiation with the histone-deacetylase (HDAC) corepressor NCoR (Yoon et al., 2003). It also has three DNA-binding zinc-finger domains at the C-terminus, and interestingly p120 is also known to bind Kaiso within this region (Daniel and Reynolds, 1999). Domain mapping indicates that p120's Arm repeats 1-7 (see Figure 1.5) are necessary and sufficient to mediate Kaiso binding

(Daniel and Reynolds, 1999). p120 also binds to cadherins via its Arm repeats, although whether cadherins and Kaiso compete for p120 binding has not so far been examined (Daniel and Reynolds, 1995).

Unlike other POZ-ZF transcription factors which recognise and bind only one specific consensus sequence, Kaiso was found to have dual-specificity DNA binding. Kaiso recognises a sequence-specific consensus sequence on DNA TCCTGCnA (known as the Kaiso binding site (KBS)) and also methylated CpG-dinucleotides (Daniel et al., 2002; Prokhortchouk et al., 2001). As predicted due to the overlapping of the p120- and DNA-binding regions of Kaiso, p120 inhibited Kaiso's DNA binding to both KBS and methyl-CpG sites (Daniel et al., 2002; Spring et al., 2005). Furthermore, p120 relieved Kaiso-mediated repression from a minimal promoter (Kelly et al., 2004; Spring et al., 2005). It had been suggested that it was possible that p120 could relieve Kaiso-mediated repression by sequestering it in the cytoplasm. However mutating the NLS of p120 prevented it from regulating Kaiso-mediated repression, suggesting that nuclear localisation of p120 was required for this effect (Kelly et al., 2004).

Several Kaiso target genes are also targets of the canonical Wnt signalling pathway, including cyclin D1, Siamois and matrilysin (matrix metalloproteinase (MMP) 7) (Park et al., 2005; Spring et al., 2005). This implicates p120 and Kaiso in the regulation of the canonical Wnt signalling pathway. Kaiso and TCF were shown to bind to each other, and combined activation of TCF by β-catenin and Kaiso derepression by p120, results in enhanced expression of Siamois (Park et al., 2005). Furthermore Kaiso and p120 regulate the expression of the non-canonical Wnt11 (Kim et al., 2004). Kaiso is also known to repress metastasis-associated protein 2 (MTA2) (Yoon et al., 2003). It is thought that Kaiso mediates transcriptional repression by recruiting a HDAC corepressor complex that includes nuclear corepressor (NcoR) (Kim et al., 2002; Prokhortchouk et al., 2001; Spring et al., 2005; Yoon et al., 2003).

It is tempting to think that β-catenin-TCF/LEF-1- and p120-Kaiso-mediated signalling pathways are highly comparable. Indeed, the finding that they

converge on a set of shared canonical Wnt gene targets adds weight to this argument and it seems that there are many parallels between the two pathways. Kaiso overexpression was shown to rescue the *Xenopus* axis-duplication phenotype caused by β-catenin overexpression and also to inhibit β-catenin-TCF/LEF-1-mediated activation of some Wnt target genes (Park et al., 2005; Spring et al., 2005). However there are some clear differences between β-catenin-TCF/LEF-1- and p120-Kaiso-mediated signalling pathways. β-catenin acts as a coactivator upon binding to TCF/LEF-1, whereas p120 appears to relieve Kaiso-mediated repression by removing it from DNA. Furthermore, β-catenin is highly unstable in the cytoplasm and requires the presence of a Wnt signal to inhibit its degradation in order to participate in nuclear signalling. In contrast p120 is not rapidly degraded in the cytoplasm and so its nuclear localisation is likely to be regulated by distinct molecular mechanisms (Thoreson et al., 2000). However a recent study has indicated that upon activation with Wnt, a dishevelled binding protein Frodo can bind to and stabilise p120 (Park et al., 2006). This suggests that activation of cells with Wnt may also enhance p120-Kaiso-mediated signalling. At present other upstream regulators of p120-Kaiso signalling are not clearly understood. Since p120 has been shown to be released into the cytoplasm upon down-regulation of cadherins at cell-cell junctions, this may also lead to an increased nuclear localisation of p120 and thus could de-repress Kaiso-regulated genes. Further study is required to understand whether cadherin function could modulate Kaiso-regulated gene repression. p120 phosphorylation could be another potential regulatory mechanism.

Many of Kaiso's gene targets (MTA2, MMP7, Siamois, cyclin D1) are linked to cell proliferation or metastasis leading to the hypothesis that Kaiso may act as a tumour suppressor. Surprisingly though, when Kaiso null mice were crossed with tumour susceptible Apc (Min/+) mice, they showed a delay in the onset of intestinal tumours (Prokhortchouk et al., 2006). Another recent study suggested that exposure of tumour cells grown *in vitro* to the tumour microenvironment (using xenograft experiments) can lead to the cytoplasmic localisation and downregulation of Kaiso expression (Soubry et al., 2005). Since p120 antagonises Kaiso-mediated repression, p120 may have an oncogenic role

through its interaction with Kaiso, in addition to its role in signalling through Rho GTPases.

A recent study by our lab showed that p120 binds a Kruppel-like transcription factor Gli-similar-2 (Glis2), which was identified in a yeast 2-hybrid screen for p120 binding partners (Hosking et al., 2007). As described above, Glis2 substantially enhances nuclear localisation of p120. p120 induces cleavage of Glis2, which is enhanced by coexpression of src (Hosking et al., 2007). Glis2 contains five tandemly repeated zinc finger domains and p120-induced cleavage removes the fifth zinc finger of Glis2, but does not prevent it from binding to DNA. Glis2 can affect neuronal differentiation in the chick neural tube, whereas the cleaved form of Glis2 requires p120 to have a similar effect (Hosking et al., 2007). Thus through binding to a second transcription factor, p120 has now also been shown to have a signalling function in neuronal differentiation.

1.4 Dynamic regulation of Adherens junctions

1.4.1 Epithelial to mesenchymal transitions

As described in section 1.1, epithelial cells tightly adhere to each other forming highly polarised sheets. Although epithelial cells are motile, in general they move within the epithelial monolayer and do not detach or move away from the monolayer. In contrast mesenchymal cells do not form an organised layer, have few intercellular contacts, and lack defined polarity (Thiery, 2003). In culture, epithelial cells grow as clusters maintaining complete cell-cell adhesion with their neighbours, whereas mesenchymal cells have a spindle-shaped, fibroblast-like morphology and are highly motile (Thiery and Sleeman, 2006). Epithelial to mesenchymal transition (EMT) is loosely defined by the disassembly of cell-cell and cell-matrix contacts leading to the loss of epithelial polarity and the separation of individual cells. This is accompanied by the acquisition of a migratory or mesenchymal-like phenotype (D'Souza-Schorey, 2005; Thiery, 2003; Thiery and Sleeman, 2006).

EMT is an important process in the tissue remodelling that occurs during embryogenesis. Without EMT, development cannot proceed past the blastula stage, which consists of an epithelial sheet surrounding a cavity known as the blastocoel (Thiery and Sleeman, 2006). EMT is crucial for the complex process of embroyonic gastrulation, which enables the transition from the blastula to a three-layered structure. In this stucture, the innermost layer is the primitive gut, known as the endoderm; the outermost layer is the epithelium that remains external, known as the ectoderm; and between the two is a looser layer of tissue composed of mesenchyme cells, known as the mesoderm (Solnica-Krezel, 2006). The formation of these three layers is vital for the formation of different organs and tissues. The formation of mesenchymal cells from the epithelial layer which then migrate into the blastocoel is vital for the production of mesoderm which in vertebrates forms skeleton, muscles, heart, kidney and may other organs (Thiery and Sleeman, 2006).

Unsurprisingly for such a vital and complex mechanism, EMT is regulated by a large number of signals such as Wnts, bone morphogenic proteins (BMPs) and many growth factors including FGF, hepatacyte growth factor (HGF), EGF and transforming growth factor (TGF)β (Blay and Brown, 1985; Boyer et al., 1989; Miettinen et al., 1994; Theriault et al., 2007; Valles et al., 1990; Wang et al., 2005; Weidner et al., 1990; Yook et al., 2006). It is difficult to follow EMT in live organisms, but the process became more amenable to experimentation in the 1980s when it was discovered that HGF, known as 'scatter factor' could convert a highly polarised epithelial cell line, Madin-Darby canine kidney (MDCK), into migratory fibroblasts by activating the tyrosine kinase receptor c-Met (Stoker et al., 1987; Stoker and Perryman, 1985). Indeed there is now a large body of evidence linking the activation of tyrosine kinase receptors by growth factors, or the activation of the tyrosine kinase Src to the induction of EMT (Behrens et al., 1993; Stoker and Gherardi, 1991; Thiery, 2003). The small GTPase Ras is activated as a result of ligand binding to RTKs, and leads to the activation of many intracellular signalling cascades including the PI3K, MAPK, and phospholipase C pathways (Downward, 2003). Ras activation itself leads to transformation and scattering of epithelial cells resulting in the disruption of cell-cell contacts, and some studies indicate that several of the pathways downstream

of Ras may be required to obtain a complete EMT (Janda et al., 2002; Potempa and Ridley, 1998; Schoenenberger et al., 1991; Thiery, 2003). As there are so many signalling pathways involved in the complex regulation of EMT, and downregulation of both cell-matrix and cell-cell adhesion occurs, sections 1.4.3 - 1.4.5 will focus on mechanisms involved in the downregulation of E-cadherin during EMT, as E-cadherin is the main cadherin expressed in epithelia and the subject of this thesis.

1.4.2 The role of E-cadherin as a tumour suppressor

The majority of human cancers (80-90%) originate from epithelial cells (Christofori and Semb, 1999). During the progression of carcinogenesis, most tumour cells deriving from epithelial tissues lose their epithelial characteristics, particularly in the transition from benign lesions to invasive metastatic cancer. This transition involves the perturbation of cell-cell adhesions and cells taking on a more migratory and invasive phenotype (Christofori and Semb, 1999; Thiery, 2002). Since the cellular organisation within tumours is highly diverse, it is hard to follow individual cells undergoing EMT. However, various carcinoma cell lines undergo partial or complete EMT *in vitro*, and a number of signalling pathways have been found to be common to EMTs in development and in tumour progression (Thiery, 2002). The putative role of EMT in the progression of carcinoma is represented in Figure 1.6.

Figure 1.6 ***EMT in tumour progression.*** *An adenoma can develop from local proliferation of a normal epithelia lined by a basement membrane, which can be further transformed to form a carcinoma in situ. In a step which may involve an EMT, local dissemination of carcinoma cells can occur producing an invasive carcinoma where the basement membrane becomes broken down. During intravasation, cells can enter lymph or blood vessels which allows their transport to secondary sites. Schematic taken from Thiery, 2002.*

It is now known that in most cancers of epithelial origin (including cancers of the breast, colon, prostate, stomach, liver, esophagus, skin, kidney and lung), E-cadherin is lost as the cancer progresses towards malignancy, and it has been proposed that this loss of E-cadherin is a prerequisite for tumour cell invasion and metastasis (Birchmeier and Behrens, 1994; Bracke et al., 1996; Christofori and Semb, 1999). Indeed, in cases of familial gastric cancers, there is evidence of germline mutations in the E-cadherin gene, suggesting that mutation of E-cadherin is sufficient to predispose individuals to this cancer (Guilford et al., 1998). As described in section 1.3.1, E-cadherin could function as a tumour suppressor by suppressing proliferation. Additionally, the role of E-cadherin in suppressing the invasive capacity of tumour cells has been shown by forced expression of E-cadherin in invasive mesenchymal tumour cells (Frixen et al., 1991; Vleminckx et al., 1991). These studies showed that E-cadherin expression prevented the invasiveness of these cells and restored their epithelial phenotype. Furthermore, a transgenic mouse model of tumourigenesis was used to show that loss of E-cadherin is a cause not a consequence of the progression from non-

invasive tumours (adenoma) to malignant, invasive tumours (carcinoma) (Perl et al., 1998). In this study, mice expressing SV40 T antigen in pancreatic β cells under the control of insulin, which reproducibly develop β cell tumours in a multistage tumourigenesis pathway, were crossed with mice expressing either wild-type or dominant negative E-cadherin specifically in pancreatic β cells. Maintainance of E-cadherin during β cell tumourigenesis resulted in the blocking of tumour development at the stage of adenoma (Perl et al., 1998). In contrast, expression of dominant negative E-cadherin resulted in early invasion and metastasis (Perl et al., 1998). This result indicates that loss of E-cadherin is a rate-limiting step in the progression from adenoma to carcinoma *in vivo*. Since blocking E-cadherin function actively enhances invasion and metastasis, it is now crucial to determine whether specific signals evoked by E-cadherin can participate in the control of invasiveness of cells.

Several studies have also indicated that during tumour progression, E-cadherin is replaced by increased expression of other 'mesenchymal' cadherins such as N-cadherin or cadherin-11, a phenomenon known as 'cadherin switching' (Christofori, 2003; Li and Herlyn, 2000; Tomita et al., 2000). Indeed, expression of N-cadherin in various cancer cell types has been shown to induce cell motility and invasiveness, thus showing the opposite effect of E-cadherin (Hazan et al., 2000; Islam et al., 1996; Li et al., 2001). At present the functional implications of cadherin switching remain incompetely understood. N-cadherin, unlike E-cadherin, promotes a more dynamic adhesion state (Christofori, 2003; Li and Herlyn, 2000; Tomita et al., 2000), and as described in section 1.3.3, N-cadherin can enhance FGF receptor signalling in neurons enhancing neurite outgrowth which is related to cell migration and invasion. However greater understanding is required to understand the relevance of cadherin switching in EMT and tumour progression.

1.4.3 Transcriptional downregulation of E-cadherin

E-Cadherin expression has been shown to be downregulated during EMT by several zinc-finger and basic helix-loop-helix (bHLH) transcriptional repressors (Peinado et al., 2007). In 2000 it was first discovered that a zinc-finger

transcriptional repressor Snail represses expression of E-cadherin through an E2-box sequence within the E-cadherin promotor (Cano et al., 2000). This study also showed that expression of Snail in epithelial cells could promote EMT and induce a fibroblastic and invasive phenotype. Furthermore it was shown that Snail is expressed in regions of mouse and human carcinomas that are E-cadherin-deficient and invasive, but absent from non-invasive carcinomas where E-cadherin levels remain high. Similarly the bHLH transcription factor E12/E47 also represses E-cadherin expression and induces EMT (Perez-Moreno et al., 2001). Slug, which is closely related to Snail, acts in a similar manner but is a less effective at repressing E-cadherin than Snail and E12/E47 (Bolos et al., 2003). Upregulation of Snail occurs in response to TGFβ, in a manner that is dependent on Ras activation of MAPK and PI3K signalling pathways (Peinado et al., 2003). Furthermore upregulation of Snail was shown to be essential for HGF-mediated scattering of HepG2 cells, since knockdown of Snail with RNAi prevented scattering (Grotegut et al., 2006). This study showed that HGF-mediated induction of Snail also involves MAPK signalling and activity of early growth response factor-1. Interestingly, the *snail* gene is repressed by metastasis associated gene 3 (MTA3), a component of the transcriptional co-repressor complex NuRD (Fujita et al., 2003). MTA3 is a vital component of a pathway activated by the estrogen receptor (ER). Breast carcinomas that are negative for ER do not express MTA3, resulting in upregulation of Snail and loss of E-cadherin (Fujita et al., 2003). Thus the loss of ER or MTA3, or the upregulation of Snail, is associated with poor prognosis in breast cancer.

1.4.4 Downregulation of E-cadherin at cell-cell contacts by endocytosis

As described in section 1.2.4, regulation of E-cadherin trafficking can influence the stability of adherens junctions. There is a wealth of evidence implicating endocytosis of E-cadherin in the downregulation of adherens junctions during EMT (D'Souza-Schorey, 2005). It was first shown by electron microscopy studies that loss of adherens junctions is accompanied by accumulation of coated vesicles (Warren and Nelson, 1987). It is now understood that cadherins undergo constitutive endocytosis and recycling, and that endocytosis is enhanced when

cell-cell contacts are disassembled, particularly in response to growth factor signalling (Kamei et al., 1999; Le et al., 1999; Wrobel et al., 2004).

The small GTPase ARF6 is a known regulator of endosome trafficking and has emerged as a regulator of E-cadherin endocytosis (Palacios et al., 2001; Palacios et al., 2002; Paterson et al., 2003). HGF stimulation of MDCK cells has been shown to activate ARF6, and dominant negative ARF6 blocks HGF-induced internalisation of cadherins (Palacios et al., 2001). As described in Section 1.3.2, Rac1 has a well established role in promoting the formation of adherens junctions. In 2003, a study was carried out following Rac1 activation over time as cell scattering was induced with HGF treatment or Src activation. It was shown that a transient decrease in Rac1 activity occurs during the disassembly of adherens junctions which is accompanied by a depolymerisation of actin at sites of cell-cell contact (Palacios and D'Souza-Schorey, 2003). This is then followed by the restoration of Rac1 activation as the cells become more migratory and produce lamellipodia (Palacios and D'Souza-Schorey, 2003). This reduction in Rac1 activity during cell scattering was shown to be dependent on ARF6 activity (Palacios and D'Souza-Schorey, 2003). It has been proposed that the loss of polymerised actin around the adherens junction at the start of scattering may 'loosen up' adherens junctions to facilitate endocytosis (D'Souza-Schorey, 2005). The biphasic activation profile of Rac1 during cell scattering may help to explain why Rac1 activation is involved in both stabilisation of adherens junctions and enhancement of migration.

The oncogene and tyrosine kinase Src is known to trigger phosphorylation of E-cadherin and β-catenin and the dissasembly of adherens junctions (Behrens et al., 1993). Following tyrosine phosphorylation of E-cadherin by Src, phosphorylated E-cadherin has been shown to bind a protein called Hakai, which then induces the endocytosis of E-cadherin (Fujita et al., 2002). Hakai means 'destruction' in Japanese and was shown in this study to be an E3-ubiquitin ligase, mediating the addition of ubiquitin to the E-cadherin complex. Ubiquitin is a small 8.5 kDa protein which has a variey of cellular roles (Pickart, 2001; Sun and Chen, 2004). The ubiquitin system and its role in the control of various cellular processes is described in more detail in section 1.6. Hakai overexpression was shown to

induce disruption of cell-cell contacts and E-cadherin internalisaion. It decreases the half-life of E-cadherin and increases the sensitivity of MDCK cells to HGF-mediated scattering (Fujita et al., 2002). Ubiquitinated E-cadherin has been shown to be targeted to lysosomes for degradation, thus ensuring that cell-cell contacts cannot reform (Palacios et al., 2005).

Recently, our lab showed that serine phosphorylation of E-cadherin by CK1 is also implicated in the control of E-cadherin endocytosis (Dupre-Crochet et al., 2007). A CK1 inhibitor was shown to stabilise cell-cell contacts. CK1 interacts and colocalises with E-cadherin, and overexpression of CK1 isoforms disrupt cell-cell contacts. Serine 846 was identified as the CK1 phosphorylation site in E-cadherin and a constitutively phosphorylated mutant at this residue was internalised more efficiently than wild-type E-cadherin (Dupre-Crochet et al., 2007). There are at least seven isoforms of CK1 which are all ubiquitously expressed in mammals, and are involved in several cellular processes including differentiation and proliferation (Knippschild et al., 2005). Indeed, as described in Section 1.3.4, CK1 positively regulates canonical Wnt signalling by phosphorylating β-catenin which promotes its ubiquitination by the APC complex. Now it has also been shown to promote the down-regulation of adherens junctions.

An important question to be answered from a signalling perspective is what happens to the other components of the adherens junctions when cadherins are internalised. Some studies suggest that at least some cadherin is internalised along with β-catenin (Akhtar and Hotchin, 2001; Ivanov et al., 2004), whereas others suggest that catenins dissociate from cadherins after cadherin internalisation (Le et al., 1999; Lu et al., 2003). If catenins are released from cadherin after internalisation they may form a 'signalling pool' in the cytoplasm. In particular, the study by Lu et al. suggests that endocytosis in response to EGF treatment may specifically enhance β-catenin-mediated signalling. However more rigorous examination is required in order to understand whether endocytosis of E-cadherin can release catenins in to the cytosol.

1.4.5 Downregulation of E-cadherin at cell-cell contacts by proteolytic cleavage

Proteolytic cleavage of E-cadherin by several proteases has been reported to be involved in the destabilisation of adherens junctions during EMT or the metastatic progression of cancers (Kuefer et al., 2003; Lochter et al., 1997; Marambaud et al., 2002; Noe et al., 2001; Rios-Doria et al., 2003). It was first discovered in 1987 that an 80 kDa E-cadherin extracellular domain fragment is released in the culture media of tumour cells (Wheelock et al., 1987). It has since been determined that E-cadherin extracellular domain is cleaved by the metalloproteinases Matrilysin-1 (MMP-7); Stromelysin-1 (MMP-3) and ADAM (a disintegrin and metalloprotease) 10 (Lochter et al., 1997; Maretzky et al., 2005; Noe et al., 2001).

MMPs are a family of proteinases that play a vital role in remodelling the cellular microenvironment during morphogenesis, wound healing and tumour progression (Lee and Murphy, 2004; Page-McCaw et al., 2007). Their main function has been historically considered to be the degradation of the ECM, but knowledge of their substrates, and therefore their critical role in regulating the response of cells to their environment, has expanded greatly over recent years (Sternlicht and Werb, 2001). At least 24 human MMP proteins have been found and collectively they have the ability to degrade all the components of the extracellular matrix. This function is known to be important to create space for cells to migrate during tumour metastasis and developmental processes. However they can also produce specific cleavage fragments from their substrates which have independent signalling activities and can modify the activity of signalling molecules and receptors (Lee and Murphy, 2004; Page-McCaw et al., 2007). Many MMPs are soluble and are released by cells into the ECM, whereas others are transmembrane proteins with an extracellular catalytic domain. Since they are so critical to the regulation of the cellular microenvironment, the activity of MMPs is tightly regulated under normal physiological conditions and their deregulation is thought to be involved in many disease states including cancer. The activities of MMPs in most normal tissues are very low which is partly due

to low expression of MMPs. Transcription of MMPs can be induced by cytokines and growth factors, as well as by some cell-cell adhesion molecules (for example MMP-9 expression is induced by intercellular adhesion molecule-1 (ICAM-1) in T lymphoma cells) and cell-matrix interactions (Nagase and Woessner, 1999). Indeed, as described in sections 1.3.4 and 1.3.5, MMPs are gene targets of both the canonical Wnt signalling pathway and Kaiso. Furthermore, the activity of MMPs is regulated by their autoinhibitory pro-domains which are destabilized or removed in order for MMPs to be active (Page-McCaw et al., 2007), and by inhibition by endogenously expressed inhibitors, tissue inhibitors of metalloproteinases (TIMPs) (Nagase et al., 2006). ADAMs are a MMP-related family of proteins which contain a metalloproteinase domain, and are either membrane-anchored or secreted (ADAMTS) (Mochizuki and Okada, 2007). ADAMs are known to cleave many transmembrane proteins, as well as ECM components, and can also bind to integrins. They are also implicated in cancer progression (Mochizuki and Okada, 2007).

In 1997, it was shown that MMP-3 activation in mammary epithelial cells leads to a stable EMT and a premalignant phenotype (Lochter et al., 1997). Cells showed an increase in invasion and a reduction in E-cadherin and β-catenin staining at cell-cell contacts. Other endogenous MMPs and keratinocyte growth factor were shown to be upregulated in this system and the phenotypic conversion continued in the absence of continued MMP-3 expression (Lochter et al., 1997). This study showed that the extracellular domain of E-cadherin was shed in cells expressing MMP-3 with the release of an 80 kDa fragment of E-cadherin into the culture medium. This was dependent on MMP activity as an MMP inhibitor blocked its production (Lochter et al., 1997). It was later shown that both MMP-3 and MMP-7 can directly cleave E-cadherin, by treating immunoprecipitates of cell lysates (immunoprecipitated with an E-cadherin antibody recognizing the cytoplasmic domain) with purified MMP proteins (Noe et al., 2001). Both MMP-3 and MMP-7 resulted in the release of E-cadherin fragments of approximately 80 kDa that could be detected by Western blotting with an antibody recognising the extracellular region of E-cadherin. A fragment of around 40 kDa remained on the beads which was detected with an antibody recognising the cytoplasmic domain (Noe et al., 2001). Moreover, the released

80 kDa fragment was shown to induce invasion and inhibit aggregation of cells, by using conditioned medium containing the released fragment (Noe et al., 2001). These effects were blocked if the E-cadherin fragment was first depleted from the medium by immunoprecipitation (Noe et al., 2001). More recently a vital role for ADAM10-mediated cleavage of E-cadherin at a similar site to MMP cleavage was shown using ADAM10 deficient fibroblasts or by knocking down ADAM10 in epithelial cells (Maretzky et al., 2005). Indeed this study showed that E-cadherin shedding was blocked in ADAM10-deficient mouse embryos, which resulted in an accumulation of full-length E-cadherin, suggesting an *in vivo* significance for ADAM10-mediated E-cadherin cleavage. Furthermore the authors showed that increased ADAM10 expression led to increased levels of cyclin D1 (Maretzky et al., 2005). The significance of this E-cadherin cleavage for the metastatic progression of prostate cancer was indicated by a study of prostate cancer tissue samples from different stages of the disease (Kuefer et al., 2003). Detection of high amounts of the 80 kDa fragment was strongly associated with advanced prostate cancer. A cDNA microarray analysis showed that several MMPs and ADAMs were also upregulated in metastatic prostate cancer (Kuefer et al., 2003). What signals are upstream of MMP-mediated E-cadherin cleavage are still under investigation but some studies indicate that apoptosis or calcium influx may be involved, at least in cell culture studies (Ito et al., 1999; Marambaud et al., 2002). Upregulation of MMP activity has been been shown in response to HGF, Ras or Snail activation (Arbiser et al., 1997; Jorda et al., 2005; McCawley et al., 1998; Shin et al., 2005; Wang and Keiser, 2000). Indeed, HGF treatment of prostate and stomach cancer cells has been reported to induce cleavage of E-cadherin by MMP-7 (Davies et al., 2001; Lee et al., 2007).

In 2002, Marambaud et al. showed that in response to treatment with staurosporine (which strongly induces apoptosis), the fragment of E-cadherin that remains in the cell following MMP-mediated cleavages is further processed by protease complex known as γ-secretase. The γ-secretase complex and its role in cellular signalling is discussed in more detail in section 1.5. In response to staurosporine treatment or other stimuli inducing cellular stress, a caspase-3-mediated cleavage of E-cadherin also occurs (Keller and Nigam, 2003; Steinhusen et al., 2001). Marambaud et al. examined the fragments to identify

the cleavage sites as shown in Figure 1.7. They termed the 38 kDa MMP cleavage product E-cad/C-terminal fragment (CTF)1, the 33 kDa γ-secretase cleavage product E-cad/CTF2, and the 29 kDa caspase-3 cleavage product E-cad/CTF3 (Figure 1.7) (Marambaud et al., 2002).

Figure 1.7 ***Cleavage products of E-cadherin produced by metalloproteinase, γ-secretase, and caspase-3 cleavages.*** *The E-cadherin cleavage sites of MMP, γ-secretase and caspase-3 were determined by Marambaud et al. in 2003. The sizes of E-cad/CTF1, E-cad/CTF2 and E-cad/CTF3 are indicated. EC1-5 denote the extracellular E-cadherin repeats. H108 and C36 are epitopes for antibodies to the extracellular and cytoplasmic domains of E-cadherin, respectively. The antibody recognising C36 is used in this thesis. TM denotes transmembrane domain. Schematic taken from Marambaud et al.,2002.*

γ-secretase-mediated cleavage of E-cad/CTF1 was first suggested by the accumulation of E-cad/CTF1 in mouse fibroblasts from knock-out mice for Presenilin-1 (PS1) which is a vital component of the γ-secretase complex (Marambaud et al., 2002). The authors then showed the accumulation of E-cad/CTF2 following treatment of an epithelial cell line A431, with either staurosporine or a calcium ionophore (which produces calcium influx which induces MMP cleavage of E-cadherin). This accumulation of E-cad/CTF2 was blocked with an inhibitor of γ-secretase. Furthermore they showed that γ-secretase-mediated cleavage is involved in the disassembly of adherens junctions in A431 cells in response to calcium ionophore (Marambaud et al., 2002). Treatment with calcium ionophore leads to a decrease in the amount of full length E-cadherin, β-catenin and α-catenin in the membrane and cytoskeletal fraction indicating the disruption of cell-cell contacts. This was concomitant with the increase of E-cad/CTF2 in the cytosolic fraction, indicating that E-cad/CTF2 becomes solubilised into the cytosol (Marambaud et al., 2002). The loss of β-

catenin and α-catenin from the membrane and cytoskeletal fraction was delayed following treatment with the γ-secretase inhibitor (Marambaud et al., 2002). Prior to this study, the same group had shown that PS1 interacts with E-cadherin at the same E-cadherin sequence which binds p120 (Baki et al., 2001). They showed that p120 and PS1 compete for binding to E-cadherin, but that PS1 promotes binding of E-cadherin to β-catenin, enhances the association of the E-cadherin/catenin complex with the cytoskeleton and increases cell-cell aggregation (Baki et al., 2001). The authors concluded that PS1 may have dual functions in its regulation of adherens junction stability. They suggest that under conditions where cell-cell adhesion is being promoted, incorporation of PS1 into the adherens junction contributes to the stabilisation of cell-cell adhesion, but under conditions where cell-cell adhesion is being down-regulated and when MMPs are active, the PS1/γ-secretase complex may faciliate cell separation by cleaving E-cad/CTF1 (Marambaud et al., 2002). Recently it has been shown that a metalloprotease toxin *B. fragilis* toxin, which is secreted by the *Bacteroides fragilis* organism that live in the colon, stimulates γ-secretase-mediated cleavage of E-cadherin, suggesting a possible role for this cleavage in disease (Wu et al., 2007).

E-cadherin is also cleaved by another protease, calpain. Calpain is a calcium-dependent protease and E-cadherin cleavage by calpain can be induced by treatment with a calcium ionophore (Rios-Doria et al., 2003). Calpain cleaves E-cadherin intracellularly producing a 100 kDa fragment which can no longer bind to p120 or β-catenin, therefore likely abrogating its adhesive function (Rios-Doria et al., 2003). In the same study it was shown using a cDNA microarray that calpain is upregulated in localised and metastatic prostate cancer compared to normal prostate tissue. The 100 kDa E-cadherin fragment was also upregulated in prostate tumours, suggesting that calpain-mediated E-cadherin cleavage may occur during progression of prostate cancer (Rios-Doria et al., 2003).

1.5 The role of metalloproteinase- and γ-secretase-mediated cleavages in cellular signalling

In this thesis, the role of the E-cadherin cleavage product (produced by cleavages by metalloproteinases and γ-secretase) in signalling is examined. In this section, the known functions of metalloproteinase and γ-secretase cleavages in signalling are discussed.

1.5.1 The γ-secretase complex

The existance of a γ-secretase proteolytic activity was first identified during research into Alzheimer's disease. One theory to explain the pathology of this disease of dementia is that it is at least partly caused by the accumulation of deposits of a peptide known as amyloid-β (Aβ) in the brains of patients (Hardy, 2006). The Aβ peptide is an integral part of the transmembrane protein amyloid precursor protein (APP). Thus it was recognised that the action of at least two proteases was required to release this peptide (Figure 1.8). It has since been determined that the extracellular domain of APP is shed by the action of a membrane-bound aspartyl protease BACE (β-site APP-cleaving protein), and that the resulting C-terminal fragment is further processed at a site within the transmembrane region by γ-secretase (Wolfe, 2006).

Figure 1.8 ***Signalling to the nucleus via γ-secretase-mediated cleavage of transmembrane proteins.*** *γ-secretase mediated cleavages (indicated by red arrow heads) and extracellular metalloproteinase cleavages (indicated by blue arrows) are known to be involved in the cleavage of amyloid precursor protein (APP). Together these cleavages lead to the release of a peptide known as Aβ which is thought to be associated with the pathology of Alzheimer's disease. Sequential cleavages by extracellular metalloproteinases and γ-secretase have been well-characterised in the Notch signalling pathway. These cleavages result in the release of intracellular domain (ICD) of Notch which translocates to the nucleus and modulates gene transcription. A similar mechanism for the release of nuclear-localising ICDs of various membrane proteins has also been established, including APP itself, as well as ErbB4, CD44, N-cadherin and γ-protocadherins. Schematic adapted from Shih and Wang, Cancer Research, 2007.*

The γ-secretase complex has a number of components, of which the first to be identified were the Presenilins, PS1 and PS2. PS mutations frequently occur in cases of familial forms of AD, and PS knockout mice showed reduced or eliminated γ-secretase activity (Herreman et al., 2000; Zhang et al., 2000). Pharmacological evidence suggested that γ-secretase action is also mediated by an aspartyl protease activity and it was later demonstrated that two conserved aspartates in the transmembrane region of PS1 are indispensable for γ-secretase activity (Wolfe et al., 1999a; Wolfe et al., 1999b). It has also been shown that PS itself is cleaved into two fragments during its maturation to an active protein, each of which contribute one aspartate residue, and that transition-state analogue γ-secretase inhibitors bind to both fragments but not the non-processed protein

(Esler et al., 2000; Li et al., 2000). Together these data provide compelling evidence that PS proteins form the active site of γ-secretase. However at least three other components of the complex have been identified (nicastrin, Aph-1 and Pen-2), and yeast reconstitution experiments have suggested that the assembly of all four of these membrane proteins is necessary and sufficient for γ-secretase activity (Edbauer et al., 2003). However the precise molecular roles of nicastrin, Aph-1 and Pen-2 are still subjects of intense reseach.

Many studies have shown that γ-secretase cleaves numerous membrane proteins and regulates their signalling activities (Figure 1.8) (Fortini, 2002; Medina and Dotti, 2003). This was first characterized by the involvement of γ-secretase in Notch signalling, the study of which played a vital role in the identification of the γ-secretase complex.

1.5.2 The Notch signalling pathway

Notch signalling is a crucial pathway in the control of cellular differentiation events during devlopment (Artavanis-Tsakonas et al., 1999). The Notch receptor is a transmembrane protein with a single transmembrane domain and is activated by binding to ligands of the Delta/Serrate/Jagged family. Activation of Notch results in transcriptional changes due to interactions with the CSL family of transcription factors (Artavanis-Tsakonas et al., 1995). In 1998, it was shown that Notch signalling required its proteolytic cleavage to release its intracellular domain (ICD) and subsequently this process was shown to be dependent on γ-secretase (De Strooper et al., 1999; Schroeter et al., 1998; Struhl and Greenwald, 1999). Although it was not possible to observe the ICD of Notch in the nucleus by immunostaining due to the low levels of cleavage, evidence of the translocation of the Notch intracellular domain into the nucleus was presented with a reporter assay using a Notch construct C-terminally tagged with GAL4-VP16 (Struhl and Adachi, 1998). It has since been shown that, similarly to APP cleavage, cleavage of Notch by γ-secretase is preceeded by removal of the Notch ectodomain by a member of the ADAM family of proteases ADAM17 (Brou et al., 2000). ADAM10 is also implicated in Notch signalling (Hartmann et al.,

2002). The parallels between APP processing and Notch signalling are shown in Figure 1.8.

1.5.3 Metalloproteinase/γ-secretase cleavages as a general mechanism for signalling from cell-surface receptors to the nucleus

γ-Secretase cleavage of several transmembrane molecules following initial cleavage by metalloproteinases has emerged as a common mechanism for signalling to the nucleus (Fortini, 2002; Medina and Dotti, 2003). In recent years, a similar mechanism to Notch signalling has also been described for APP itself, ErbB4, CD44 as well as N-cadherin and γ-protocadherins (Figure 1.8) (Gao and Pimplikar, 2001; Haas et al., 2005; Hambsch et al., 2005; Marambaud et al., 2003; Ni et al., 2001; Okamoto et al., 2001; Shoval et al., 2007; Uemura et al., 2006a). For all these transmembrane molecules, intracellular domain fragments resulting from γ-secretase cleavage are translocated to the nucleus and have signalling functions (Figure 1.8).

The first example of a signalling function of a γ-secretase cleavage product of a cadherin protein came in a study which showed that NMDA-receptor agonists enhance γ-secretase-mediated cleavage of N-cadherin, and that the N-cadherin cleavage product N-cad/CTF2 binds and regulates the CREB binding protein (Marambaud et al., 2003). Binding of N-cad/CTF2 was shown to enhance the proteasomal degradation of CREB binding protein, thus inhibiting CRE-dependent transcription. Two further studies have shown that N-cad/CTF2 localises to the nucleus of neurons and neural crest cells (Shoval et al., 2007; Uemura et al., 2006a). NMDA receptor stimulation in neurons was also shown to induce production of N-cad/CTF2, which localised to the the nucleus when overexpressed in neurons (Uemura et al., 2006a). Furthermore this study showed that N-cad/CTF2 could inhibit β-catenin phosphorylation by GSK3β and enhance cellular levels of β-catenin (Uemura et al., 2006a). Moreover it was shown that overexpression of N-cad/CTF2 leads to enhancement of β-catenin nuclear localisation but only in isolated cells with few cell-cell contacts, as well as increased transcription of β-catenin itself and reduced transcription of N-

cadherin. Increased levels of cyclin D1 protein were also detected (Uemura et al., 2006a).

Sequential cleavage of N-cadherin by metalloproteases followed by γ-secretase has been demonstrated (Uemura et al., 2006b). This study showed that production of N-cad/CTF2 by treatment with a calcium ionophore could be blocked by addition of an inhibitor of metalloproteases. Furthermore an N-cadherin construct lacking the ectodomain produced substantial amounts of N-cad/CTF2 even in the absence of calcium ionophore, thus suggesting that supply of substrate to γ-secretase is crucial in regulation of γ-secretase-mediated cleavage (Uemura et al., 2006b). ADAM10-mediated cleavage of N-cadherin in neural crest cells has been shown to be induced by bone morphogenic protein (BMP) 4 (Shoval et al., 2007). BMP4 induces EMT of the neural crest, which is prevented by treatment with a specific inhibitor of ADAM10. The γ-secretase cleavage product of N-cadherin N-cad/CTF2, assumed to be the end product of ADAM10-mediated cleavage, was ectopically expressed in neural crest cells and rescued their migration in the presence of the specific ADAM10 inhibitor (Shoval et al., 2007). Thus N-cad/CTF2 shows antagonistic properties to full-length N-cadherin which inhibits EMT. N-Cad/CTF2 localises in the nucleus of neural crest cells and both β-catenin and cyclin D1 mRNA levels were enhanced by N-cad/CTF2 expression (Shoval et al., 2007).

Enhancement of β-catenin-mediated nuclear signalling by nuclear N-cad/CTF2 was suggested by these two studies (Shoval et al., 20007; Uemura et al., 2006a). This is surprising since as described in section 1.3.4, cadherins and TCF/LEF-1 bind to the same region of β-catenin. There are a number of possible explanations. Firstly it has been shown that β-catenin phosphorylation by GSK3β is inhibited by N-cad/CTF2, suggesting that its stability may be enhanced by production of this cleavage product (Uemura et al., 2006a). Both studies indicate transcriptional upregulation of β-catenin itself by N-cad/CTF2. It is also possible that gene products such as cyclin D1 may be upregulated by alternative signalling pathways induced by N-cad/CTF2. Further research in this area is required to fully understand these observations.

The cytoplasmic domain of γ-protocadherins also localises in the nucleus following cleavages by metalloproteinases and γ-secretase (Haas et al., 2005; Hambsch et al., 2005). Furthermore the cytoplasmic domain of γ-protocadherins was also shown to activate the promoter of γ-protocadherin locus (Hambsch et al., 2005). Despite this evidence linking cleavage of N-cadherin and γ-protocadherins to signalling functions in the nucleus, a signalling function for the cleaved cytoplasmic domain of E-cadherin has not yet been described.

1.5.4 Regulation of γ-secretase

The regulatory mechanisms of γ-secretase have not been fully characterised and therefore the regulation of the cleavage-mediated signalling pathways described in Section 1.5.3 is still not completely understood. As discussed, supply of substrate may be crucial in regulating the activity of γ-secretase. In all the signalling pathways described in Section 1.5.3, cleavage by metalloproteases to remove the ectodomain has been shown to be involved. In the case of N-cadherin, prior cleavage by metalloproteases is required for γ-secretase cleavage (Uemura et al., 2006b). Indeed a study in 2000 suggested that the main factor influencing γ-secretase cleavage of APP and Notch is the length of the short extracellular stalk remaining after metalloproteinase cleavage rather than any specific amino acid sequences (Struhl and Adachi, 2000). This study showed that artificial substrates with extracellular segments exceeding 300 amino acids are extremely poorly cleaved by γ-secretase and that those with progressively shorter segments are cleaved more efficiently, with those with less than 50 amino acids being cleaved optimally (Struhl and Adachi, 2000). This suggests that the requirement for prior removal of the ectodomain of transmembrane proteins may be a general mechanism for regulating γ-secretase-mediated cleavage. This idea was challenged by a study in *Drosophila* which showed that metalloproteinases could cleave Notch at two nearby sites producing very similar membrane anchored C-terminal fragments, but only one of these could be cleaved by γ-secretase (Lieber et al., 2002). However, it seems that metalloprotease cleavage is required prior to γ-secretase cleavage of transmembrane proteins, although it

remains to be determined whether this is due to a general requirement for removal of bulky extracellular domain, or due to the production of specific cleavage sites.

γ-secretase may also be regulated by assembly of the complex itself. As described in Section 1.5.1, PS itself must be cleaved by an as yet unidentified presenilinase. Furthermore, most newly synthesized PS is rapidly degraded, and exogenous PS has been shown not to increase levels of the processed PS fragments, suggesting that PS maturation by cleavage is tightly regulated by competition for unknown limiting cellular factors (Medina and Dotti, 2003; Thinakaran et al., 1997). Nicastrin has also been implicated in the stabilization and maturation of PS (Medina and Dotti, 2003). Moreover since Nicastrin also binds the C-terminal membrane-anchored substrates of γ-secretase, it has been suggested that Nicastrin may be involved in sensing the size of the extracellular domain of substrates if this is indeed critical in determining cleavage efficiency (Medina and Dotti, 2003). Understanding the roles of the other components of the γ-secretase complex is likely to provide greater insight into its regulation.

It is also considered that control of γ-secretase localisation in the cell may regulate its activity (Fortini, 2002; Medina and Dotti, 2003; Uemura et al., 2007). Althogh most PS is found in the ER and Golgi, endogenous PS1 has been reported to localise at the plasma membrane as an active molecule (Chyung et al., 2005). As described in section 1.4.5, PS binds to cadherins and it has also been shown that PS binds to PI3K and promotes activation of PI3K by increasing the cadherin/PI3K association (Baki et al., 2004). A recent paper showed that expression of N-cadherin in CHO (chinese hamster ovary) cells (which express very low levels of endogenous cadherins), and the concomitant formation of cell-cell contacts, lead to an increase in cell surface levels of γ-secretase (indicated by the presence of both PS and nicastrin at the cell surface) (Uemura et al., 2007). GSK3β was also shown to phosphorylate PS and inhibit its association with N-cadherin, thereby down-regulating the cell surface levels of PS (Uemura et al., 2007). A model has been proposed whereby N-cadherin-mediated cell-cell contacts recruit γ-secretase to the cell surface. This may be important for γ-

secretase to cleave cell surface molecules and activate signalling pathways as described in section 1.5.3. Furthermore, the recruitment of γ-secretase may also enhance signalling via PI3K. GSK3β-mediated phosphorylation of PS may play a role in down-regulating these pathways.

Despite reports of active γ-secretase at the cell surface, the site of γ-secretase-mediated cleavage of transmembrane proteins during signalling is not clear. It was shown in 2004 that endocytosis of Notch may be required for its cleavage by γ-secretase (Gupta-Rossi et al., 2004). This study used a Notch construct that is similar to the product of metalloprotease-mediated cleavage, which is expressed at the plasma membrane and is constitutively cleaved by γ-secretase. Blocking endocytosis with dominant negative forms of Dynamin or Eps15 inhibited γ-secretase-mediated cleavage of this construct (Gupta-Rossi et al., 2004). Monoubiquitination of Notch at a specific lysine residue was also shown to occur in this study, and mutating this residue inhibited both internalization and γ-secretase-mediated cleavage of Notch (Gupta-Rossi et al., 2004). Furthermore it has been shown that the γ-secretase complex is found in the lysosome and is activated by low pH (Pasternak et al., 2003). The idea that endocytosis may enhance γ-secretase activity was given further support by a study showing that activation of the β2-Adrenergic receptor leads to the endocytosis of γ-secretase to late endosomes and lysosomes where there was increased cleavage of APP (Ni et al., 2006). In this study blocking transport from early to late endosomes inhibited γ-secretase activity. Together, these studies raise the possibility that endocytosis and ubiquitination may be involved in regulating γ-secretase cleavage of its other substates. Clarifying the cellular compartments in which γ-secretase cleaves its substrates may improve understanding of its regulation.

1.6 Ubiquitination in signalling

In Chapter 5 of this thesis, ubiquitination and phosphorylation of cleaved E-cadherin are investigated. Phosphorylation is extremely well characterized as a covalent modification controlling signalling functions of proteins. There are

many specific protein kinases which catalyse the addition of the phosphate group to serine, threonine and tyrosine residues of proteins. The addition of the phosphate group can act as a docking site for the recruitment of other proteins, or may activate or inactivate the active sites of enzymes. Indeed, the activation of other kinases by phosphorylation can lead to a signalling cascade, the control of which allows fine tuning of signalling pathways in time and space, for example in MAPK signalling pathways (Morrison and Davis, 2003). However the roles of ubiquitination in signalling are less well characterised. This section briefly describes what is understood about the ubiquitin system and its varied roles in cell biology.

1.6.1 The ubiquitin system

Ubiquitin is a small 76 amino acid conserved protein of around 8.5 kDa which is ubiquitously expressed in all eukaryotic cells. Ubiquitination of a protein usually occurs through the formation of a bond between the C-terminal glycine residue of ubiquitin and the ε-amino group of a lysine residue of the protein substrate (Pickart, 2001). This reaction requires three enzymes acting in a sequential fashion: a ubiquitin activating enzyme (E1) which forms a thiol ester bond with the carboxyl group of the C-terminal glycine of ubiqutin; a ubiquitin conjugating enzyme (E2) which transiently carries the activated ubiquitin molecule through a thiol ester bound via the active site cysteine residue of the E2; and a ubiquitin ligase (E3) that transfers the activated ubiquitin to the substrate lysine residue (Pickart, 2001). Recognition of substrates is mediated by presence and accessibility of motifs in the substrate which are recognised by E3 ubiquitin ligases. E3 ubiquitin ligases are therefore the specificity determinants in the system (Laney and Hochstrasser, 1999). E3 ubiquitin ligases consist of two protein families: those that contain HECT domains and those that contain RING domains. Although there is little sequence or structural similarity between HECT and RING domains, E2 conjugating enzymes can interact with both types of E3 ligases (Pickart, 2001). It is known that HECT domains contain a conserved cysteine residue which forms a thiol ester during catalysis. The RING finger domain has a set of cysteine and histidine residues which are ligands to two zinc

ions. It is thought that RING finger E3 ligases may form a scaffold to link E2 ligases to substrates (Pickart, 2001). Phosphorylation of substrates has emerged as a common signal to trigger their ubiquitination (Pickart, 2001). Indeed, as described in section 1.3.4, phosphorylation of β-catenin by CK1 and GSK3β is known to trigger its ubiquitination.

1.6.2 Multiple cellular roles of ubiquitination

Ubiquitination of a substrate frequently stops after the addition of one ubiquitin molecule (monoubiquitination) or further ubiquitin molecules may be added to the lysine residues of ubiquitin molecules already added to the substrate to form a chain (polyubiquitination) (Pickart and Fushman, 2004). Ubiquitin conjugation to form chains can occur on a number of lysine residues within the ubiquitin molecule. Polyubiquitination through lysine 48 residues in ubiquitin are the most well characterized and are well known to be involved in targeting the substrate protein for degradation in the proteasome (Pickart and Fushman, 2004). Indeed, it has been shown that a single lysine-48 linked chain is sufficient to target a model substrate to the proteasome (Chau et al., 1989). Monoubiquitination has emerged as having differerent roles from lysine-48 linked polyubiquitin and has been shown to be involved in endocytosis of transmembrane proteins (including both E-cadherin and Notch as described in Sections 1.4.4 and 1.5.4), as well as DNA repair and nuclear localisation (Li et al., 2003; Sun and Chen, 2004).

More recently it has emerged that lysine-63 linked polyubiquitin chains also have varied cellular roles in DNA damage tolerance, signalling pathways by activating a kinase important in the inflammatory response, protein trafficking and nuclear import of proteins (Geetha et al., 2005; Pickart and Fushman, 2004). An NMR study has shown that lysine-48- and lysine-63-linked ubiquitin chains form structurally different conformations (Varadan et al., 2004). Ubiquitin chains linked through lysine-48 form a closed conformation with the hydrophobic residues forming the interface between the ubiquitin monomers, whereas lysine-63 linked ubiquitin chains form an extended conformation with no direct interaction between their hydrophobic surfaces (Varadan et al., 2004). It is

suggested that the exposure of hydrophobic surfaces in lysine-63 linked chains may provide docking sites for proteins which may explain its more varied functions in signalling. Polyubiquitin chains linked through lysine-6 residues have been shown to be produced by an E3 ubiquitin ligase formed by the tumour suppressor BRCA1 when complexed to another protein BARD1. Lysine-6-linked ubiquitin chains which have been shown to be recognised by the proteasome but do not trigger degradation (Nishikawa et al., 2004). Indeed these lysine-6-linked ubiquitin chains have been implicated in DNA repair (Morris and Solomon, 2004). Other polyubiquitin chains are less well characterized but lysine-11-linked chains have been shown to signal proteasomal degradation *in vitro* (Pickart and Fushman, 2004). Moreover, in yeast, addition of a lysine-48 linked polyubiquitin chain can lead to inhibition of a transcription factor Met4 without inducing degradation (Flick et al., 2004). This suggests that even lysine-48 linked ubiquitin chains may have signalling functions beyond their role in targeting proteins to the proteasome.

Small ubiquitin-like modifiers (SUMO) are a family of proteins which are highly related to ubiquitin. SUMO proteins are larger than ubiquitin at around 12 kDa and SUMOylation of substrates occurs by an analogous conjugation method to ubiquitination (Johnson, 2004). SUMO proteins are also conjugated to the lysine residues of substrates but the enzymes of the SUMO pathway are specific. In contrast to ubiquitin, most SUMOylated lysine sites *in vivo* have only one SUMO monomer, but poly-SUMO chains have been observed (Johnson, 2004). SUMOylation does not appear to be involved in protein degradation but has been shown to trigger nuclear localisation of proteins, as well as having roles in transcriptional regulation, maintenance of genome integrity, and signal transduction (Seeler and Dejean, 2003).

1.6.3 Ret finger protein, a RING finger domain containing protein

In this thesis, the identification of Ret finger protein (RFP) as a novel binding partner of p120 is described. RFP is a member of an expanding family of RBCC (also called TRIM) proteins which all contain a tripartite motif comprising a

RING finger domain, a B-box domain and a coiled-coil domain (Meroni and Diez-Roux, 2005). B-box domains are also zinc-binding domains, and although no specific function has been attributed to them, it is thought that they are involved in protein-protein interactions, as is the case for the coiled-coil domain. A growing number of RBCC/TRIM family members have been shown to have E3 ubiquitin ligase activities (Meroni and Diez-Roux, 2005). The biological function of RFP is still incompletely understood. In this thesis, evidence that RFP is involved in ubiquitination of cleaved E-cadherin is presented. The remainder of this section will summarise what is currently known about the function of RFP.

RFP was originally identified as the N-terminal half of a fusion protein with the RET tyrosine kinase which posseses transforming activity (Takahashi et al., 1988). RFP is widely expressed in tissues and its nuclear localisation has been reported, but its cytoplasmic or nuclear localisation appears to depend on cell type. Indeed RFP has been shown to contain a functional NES and activation of protein kinase C leads to nuclear export of RFP (Harbers et al., 2001). It has also been reported to be associated with the nuclear matrix and to be present in nuclear bodies known as PML nuclear bodies (Cao et al., 1998; Isomura et al., 1992). RFP binds and modulates the activity of several transcription factors including Enhancer of Polycomb and also inhibits the activity of Retinoblastoma protein (Krutzfeldt et al., 2005; Shimono et al., 2000). Several studies indicate that RFP acts as a transcriptional repressor including repressesing transcriptional activation by bHLH transcription factors (Bloor et al., 2005; Matsuura et al., 2005; Shimono et al., 2000). However, it is thought that RFP may have dual transcriptional functions and could participate in the activation of transcription when in complex with some transcription factors (Shimono et al., 2005; Shimono et al., 2000; Townson et al., 2006). Indeed, in a recent study, RFP was shown to be necessary for estrogen receptor-mediated expression of cyclin D1, and using chromatin immunoprecipitation (ChIP), it was found to bind the promotor region of an estrogen receptor regulated gene (Townson et al., 2006). DNA binding of RFP has also been shown in an *in vitro* DNA binding assay (Isomura et al., 1992). Although no clear picture of the biological role of RFP has emerged, it

seems likely that RFP is involved in regulation of transcription of several genes and may have both activating and repressive functions.

1.7 Aims of the project

The broad aim of this thesis project is to add to the understanding of the mechanisms of signalling pathways induced by E-cadherin. In particular the aim is to determine whether there is a signalling role for γ-secretase-mediated cleavage of E-cadherin (as has been shown for other transmembrane proteins, described in section 1.5.3), in addition to its role in down-regulating adherens junctions.

Specific aims of this project:

- To investigate the stimuli inducing production of the E-cadherin cleavage product E-cad/CTF2 (Chapter 3)
- To determine whether E-cad/CTF2 localises to the cell nucleus and what regulates its nuclear localisation (Chapters 4 and 5)
- To examine the roles of E-cad/CTF2 in the nucleus (Chapter 6).

CHAPTER 2

2 MATERIALS AND METHODS

2.1 Molecular Biology

2.1.1 Polymerase Chain Reaction

PCR reactions were performed in a final volume of 100 µl containing 10 ng template DNA (2 µl of a 5 ng/µl stock), 0.2 mM dNTPs (8 µl from a 2.5 mM stock solution) (Amersham), 1 µl Vent polymerase (New England Biolabs), 10 pmol of each oligonucleotide primer (1 µl of a 100 µM stock) (MWG Biotech) and 10 µl Vent polymerase 10x buffer (NEB). DNA was amplified in a thermocycler (Jencons-PLS) as follows: 1x at 95°C for 5 min, 30-35x [95°C for 1 min, 52-55°C for 1 min, 72°C for 2 to 3 min] and 1x 72°C for 10 min. PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen) according to the manufacturers instructions.

2.1.2 DNA restriction digests

Plasmid DNA and PCR products (2.5-10 µg DNA per reaction) were were mixed with 2 µl restriction enzyme buffer (NEB), 2 µl of 10x Bovine Serum Albumin (BSA, NEB), and 1 µl (10 - 20 units) of each restriction enzyme (NEB) in a final volume of 20 µl using distilled H_2O (dH_2O) and were incubated for 1 hr at either 25°C or 37°C. Reaction mixes were prepared as indicated in Table 2.1:

Type	Mini-prep analysis	Cloning (vector)	Cloning (Insert)
Amount of DNA	2.5-5 µg	5-10 µg	10 µg
H_20	10.5 µl	11 µl	7 µl
10x BSA	2 µl	2 µl	2 µl
10x Buffer	2 µl	2 µl	2 µl
DNA	5 µl	4 µl	8 µl
Enzyme (10-20 units)	1 µl	1 µl	1 µl

Table 2.1 *Details of restriction digest reactions used for mini-prep analysis and cloning of the constructs prepared in this thesis.*

Cut vectors were further treated with Alkaline Phosphatase (Roche). 113 µl H_2O, 15µl AP buffer (Roche), and 2 µl AP were added to the 20 µl vector restriction digest mixture, and incubated at 37°C for 1 h. 1 µl of 0.5 M EDTA was then added to the mixture and the reaction was heated at 75°C for 10 min.

2.1.3 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate DNA samples at 100 volts for 1 hour. Gels were made up using 1% agatose in TAE (Tris-acetate 50x stock: 2 M Tris Acetate 100 mM Na_2EDTA (National Diagnostics), diluted with dH_2O). Ethidium bromide was added to the gel to a final concentration of 0.5 µg/ml in order to detect DNA under UV light. DNA samples were mixed with 6 x DNA loading buffer (0.25% Xylene cyanole (w/v), 0.25% Bromophenol blue (w/v), 40% sucrose (w/v) made up in dH_2O). DNA fragment sizes were estimated by comparing with migration of bands from the 1Kb DNA ladder (Promega). The QIAquick® Gel Extraction Kit (Qiagen) was used to extract and purify DNA bands from agarose gels, according to the manufacturers instructions.

2.1.4 DNA ligation reactions

Ligation reactions were carried out using the reaction mixes detailed in Table 2.2:

Type	Vector Ligation Control	Ligation	Insert Ligation Control
Vector (µl)	2	2	-
Insert (µl)	-	7	7
10x ligation buffer (µl)	2	2	2
ATP (10 mM) (µl)	1	1	1
T4 DNA ligase (µl)	1	1	1
H_2O (µl)	14	7	9

Table 2.2 *Details of ligation reactions used in the preparation of constructs in this thesis.*

Reactions were carried out using 10x DNA ligase buffer (Roche) and T4 DNA ligase (5 U/µl, Roche). Reaction mixes were incubated overnight at 16°C. For

blunt end ligations,1μl T4 DNA Polymerase (Roche) and 2μl dNTP were added to the restriction digest mixture, and incubated at 37°C for 30 min. The reaction mixture was then heated at 75°C for 10 min, and ligation reactions were carried out as detailed in table 2.2.

2.1.5 Preparation of competant *E. coli.*

The XL-1 Blue strain of *E. coli* was streaked on an LB agar plate and grown overnight at 37°C. 3 ml of L-Broth (Luria-Bertani medium: 1% bacto-tryptone (DIFCO laboratories), 0.5% bacto-yeast extract (DIFCO laboratories), 1% sodium chloride made up in de-ionised water) was inoculated with a single colony from the plate. After overnight growth at 37°C with agitation this culture was used to inoculate 250 ml of SOB (2% bacto-tryptone (w/v), 0.5% bacto-yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, pH 6.7 – 7.0). The SOB culture was grown at 18°C with agitation until an OD_{600} reading of approximately 0.6 was obtained. The culture was placed on ice for 10 min and then centrifuged at 4°C for 10 min at 2500 x g. The pellet was resuspended in 80 ml of ice-cold TB (10 mM Pipes, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl, pH 6.7) and placed on ice for 10 min. Following centrifugation at 4°C for 10 min at 2500 x g, bacteria were then gently resuspended in 20 ml ice-cold TB. Dimethyl sulfoxide (DMSO, Sigma) was added to a final concentration of 7% and cells were placed on ice for a further 10 min. 500 μl aliquots were frozen in liquid nitrogen and stored at -80°C. Competency was tested by transforming cells with pBluescript vector. Briefly, cells were transformed by heat shock (see section 2.1.7) with 0.1 ng and 0.001 ng of pBluescript. A high level of competency was indicated by the growth of 10,000 and 1,000 colonies respecitively.

2.1.6 *E.coli* transformation by 'heat shock'

Competent *E.coli* were thawed on ice. DNA to be transformed was placed in 1.5 ml microfuge tubes (1 μl of plasmids for amplification, 10 μl of ligation mixtures) and mixed with 50 μl competent cells and placed on ice for 5 min. The

cells-DNA mix was incubated at 42°C for 45 sec then immediately returned to ice for 5 min. 200 µl of SOC medium (Invitrogen) was added and the cells were incubated with moderate agitation for 10 min (amplification) or 30 min (ligation). 200 µl of transformed bacteria were plated onto LB agar plates containing either ampicillin (50 µg/ml) or kanamycin (30 µg/ml) for selection and incubated overnight at 37°C.

2.1.7 DNA mini preps from bacterial cultures

3 ml of LB containing appropriate antibiotics were inoculated with individual bacterial colonies and grown overnight with agitation at 37°C. Cultures were centrifuged at 5000 x g for 30 sec. Supernatants were removed and pellets were resuspended in 400 µl TENS buffer (1 mM Tris/Cl pH 7.5, 1 mM EDTA pH 8, 0.1N NaOH, 0.5% SDS). Tubes were gently inverted five times. 200 µl of 3M NaOAc, pH 5.2 was added to each tube and mixed thoroughly. The solutions wer centrifuged at 5000 x g for 10 min, and supernatants (approx. 600 µl) were transferred to clean tubes. 600 µl of isopropanol (BDH laboratory supplies) was added to each tube, then the solutions were vortexed and centrifuged at 5000 x g for 10 min. The supernatant was removed and the pellets were rinsed carefully with 70% ethanol. DNA pellets were left to air dry for 10 min then resuspended in 40 µl dH_2O with 10 µg RNase A and incubated at 37°C for 10 min. 5 µl of each preparation was examined by restriction enzyme digestion, as detailed in table 2.1.

2.1.8 DNA maxi preps from bacterial cultures

Maxi preps of DNA were prepared using GenElute HP Plasmid Maxiprep Kit ® (Sigma) according to the manufacturers instructions. DNA was eluted using 5 ml of dH_2O pre-warmed to 37°C, then ethanol (EtOH) precipitated by adding 500 µl of 3M NaOAc, pH 5.2 and 12.5 ml of 100% EtOH and centrifuging at 5000 x g for 30 min at 4°C. Supernatants were discarded and pellets were resuspended in 1 ml 70% ice-cold EtOH and centrifuged at 5000 x g for 10 min. Pellets were left to air dry and resuspended in 500 µl of dH_2O.

2.1.9 Determination of DNA concentration

The concentration and purity of DNA maxi preps and mini preps were examined by spectrophotometry ($Abs_{260/280}$) and by comparison with standards using gel electrophoresis, respectively.

2.1.10 Site directed mutagenesis

Amino acid changes were carried out using the Quikchange® Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturers instructions using the primers outlined in table 2.6.

2.1.11 DNA constructs used for experiments in this thesis

Table 2.3 describes constructs used in this study that were prepared by other members of the Fujita lab.

Construct	Vector	Insert
pcDNA-HA-E-cad/CTF2	pcDNA-HA	E-cadherin (cytoplasmic domain), mouse
This construct was produced by Dr. Yasuyuki Fujita		
pcDNA-FLAG-p120	pcDNA-FLAG	p120 isoform 1B, full-length, mouse
This construct was produced by Dr. N. Serpente		
pcDNA-HA-p120	pcDNA-HA	p120 isoform 1B, full-length, mouse
This construct was produced by Dr. N. Serpente		
pcDNA-FLAG-Hakai	pcDNA-FLAG	Hakai, full-length, mouse
This construct was produced by Dr. N. Serpente		
pcDNA-FLAG-RFP	pcDNA-FLAG	Ret Finger Protein, full-length, mouse
This construct was produced by Dr. N. Serpente		
pBSSR-HA-Ub	pBSSR	Ubiquitin, full-length, mouse
This construct was produced by Dr. Y. Fujita		
pCW7-His-Ub	pCW7	Ubiquitin, full-length, mouse
This construct was produced by Dr. Y. Fujita		

pBTM-ECad1	pBTM	E-cadherin, amino acids 734 – 822, mouse
This construct was produced by Dr. Y. Fujita		
pBTM-ECad2	pBTM	E-cadherin, cytoplasmic domain (amino acids 734 – 884), mouse
This construct was produced by Dr. Y. Fujita		
pBTM-ECad3	pBTM	E-cadherin, amino acids 771 - 822, mouse
This construct was produced by Dr. Y. Fujita		
pBTM-ECad4	pBTM	E-cadherin, amino acids 771 - 884, mouse
This construct was produced by Dr. Y. Fujita		
pBTM-ECad5	pBTM	E-cadherin, amino acids 771 - 848, mouse
This construct was produced by Dr. Y. Fujita		
pcDNA-HA-ubiquitin (K 11, 27, 29, 48, 63 R)	pcDNA3.1	Full length of mouse ubiquitin amplified from pBSSR-HA-ubiquitin with all lysine residues mutated to arginine.
This construct was produced by Dr. Y. Fujita		
pcDNA-HA-ubiquitin (K48R)	pcDNA3.1	Full length of mouse ubiquitin amplified from pBSSR-HA-ubiquitin with lysine 48 mutated to arginine.
This construct was produced by Dr. Y. Fujita		
pcDNA-HA-ubiquitin (K63R)	pcDNA3.1	Full length of mouse ubiquitin amplified from pBSSR-HA-ubiquitin with lysine 63 mutated to arginine.
This construct was produced by Dr. Y. Fujita		

Table 2.3 *Details of constructs used during this research that were obtained from other members of the Fujita Lab.*

Table 2.4 describes constructs used during this research which were provided by other members of the research community.

Construct	Source
pGL3-control vector	Kindly donated by Dr. Carien Niessen
pGL3-4xKBS	Kindly donated by Dr. Carien Niessen
pLK-E-cadherin (762AAA764)	Kindly donated by Dr. Carien Niessen
pME18S-FLAG-ADAM10	Kindly donated by Dr. Eiichiro Nishi
pTOP-FLASH	Kindly donated by Prof. Walter Birchmeier
pFOP-FLASH	Kindly donated by Prof. Walter Birchmeier
pcH110-LacZ	Kindly donated by Prof. Walter Birchmeier
pcDNA-myc-β-catenin	Kindly donated by Prof. Walter Birchmeier
pFA-CMV	Kindly donated by Dr. Julie Pitcher
pFR-luciferase (firefly)	Kindly donated by Dr. Julie Pitcher
pCMV-Renilla	Kindly donated by Prof. Walter Birchmeier
pcDNA-myc-Kaiso	Kindly donated by Dr. Juliet Daniel
pGL3-Basic-2.3kb-HMat	Kindly donated by Dr. Juliet Daniel
pGL3-myc	Kindly donated by Dr. Hiroyoshi Ariga
pGL3-cyclin D1	Kindly donated by Dr. Hiroyoshi Ariga

Table 2.4 *Constructs used in this research provided by other members of the research community.*

Table 2.5 describes a series of constructs prepared by PCR and subcloning.

Construct	Vector	PCR Template	Enzymes used
E-cad/CTF2	pcDNA3.1	pBAT-E-cadherin	BamHI/NotI
Primer 1: 5'GGAATTCGCGGCCGCGGTACCATGCGGAGGAGAACGGTGGTC 3' **Primer 2:** 5'GGAATTCGGATCCTAGTCGTCCTCGCCACC3' Amplification of the enire cytoplasmic domain of mouse E-cadherin (amino acids 734 – 884)			
E-cad/CTF3	pcDNA3.1	pBAT-E-cadherin	BamHI/NotI
Primer 1: 5'ATTCGCGGCCGCGGTACCATGAATGTGTATTACTATGATGAAGAAGGAGG3' **Primer 2:** 5'GGAATTCGGATCCTAGTCGTCCTCGCCACC 3' Amplification of mouse E-cadherin amino acids 753 - 884			
E-cad/CTF2(AAA)	pcDNA3.1	pLK-Ecadherin (762AAA764)	BamHI/NotI
Primer 1: 5'GGAATTCGCGGCCGCGGTACCATGCGGAGGAGAACGGTGGTC 3' **Primer 2:** 5'GGAATTCGGATCCTAGTCGTCCTCGCCACC3' Amplification of entire cytoplasmic doman of human E-cadherin (with triple alanine mutation at residues 762 – 764)			
E-cad/CTF2-NLS-myc	pCMV-myc-nuc (Invitrogen)	pBAT-E-cadherin	NotI
Primer 1: 5'GGAATTCGCGGCCGCGGTACCATGCGGAGGAGAACGGTGGTC 3' **Primer 2:** 5'GGAATTCGGATCCTAGTCGTCCTCGCCACC3' Amplification of the enire cytoplasmic domain of mouse E-cadherin (amino acids 734 – 884). Constructed jointly with Anthony Wadlow			
E-cad/CTF2 (AAA)-NLS-myc	pCMV-myc-nuc (Invitrogen)	pLK-Ecadherin (762AAA764)	NotI
Primer 1: 5'GGAATTCGCGGCCGCGGTACCATGCGGAGGAGAACGGTGGTC 3' **Primer 2:** 5'GGAATTCGGATCCTAGTCGTCCTCGCCACC3' Amplification of entire cytoplasmic doman of human E-cadherin (with triple alanine mutation at residues 762 – 764) . Constructed jointly with Anthony Wadlow			
GFP-E-cad/CTF2-NLS	pcDNA/TO/GFP	pcDNA-E-cad/CTF2-NLS	EcoRI/Xho1
Primer 1:			

5'CGGCGCCTCGAGGCGGAGGAGAACGGTGGTC**3'** **Primer 2:** **5'**CGGCGCCGAATTCCTATGCGGCCCCATTCAGATCC**3'** Amplification of the enire cytoplasmic domain of mouse E-cadherin (amino acids 734 – 884). Kindly constructed by Anthony Wadlow			
pCAN-Myc-CK1α	pCAN-myc	pCS2-CK1α	BamHI/NotI
Primer 1: **5'**CGAATTCGGATCCGCATGGCGAGCAGCAGCGGCTCC**3'** **Primer 2:** **5'**GGAATTCGCGGCCGCTTAGAAACCTGTGGGGGTTTGGGCC**3'** Amplification of full length Rat CK1α			
pCAN-Myc-CK1ε	pCAN-myc	pCS2-CK1ε	ClaI/NotI
Primer 1: **5'**CATCGATGTCGACACATGGAGCTGAGAGTGGGGAAC**3'** **Primer 2:** 5'GGTCGACGCGGCCGCTCATTTCCCAAGGTGGTCAAATGGC3' Amplification of full length *Xenopus laevis* CK1ε			

Table 2.5 Constructs prepared by PCR amplification and subcloning.

Table 2.6 describes constructs that were prepared by site-directed mutagenesis.

Construct	Template	Primers
pcDNA-E-cad/CTF2(K740R)	pcDNA-E-cad/CTF2 (Lysine → Arginine)	**Primer 1:** **5'**GGAGAACGGTGGTCAGAGAGCCCCTGCTGCC**3'** **Primer 2:** **5'**GGCAGCAGGGGCTCTCTGACCACCGTTCTCC**3'**
pcDNA-E-cad/CTF2(K740R, K818R)	pcDNA-E-cad/CTF2 (K740R) (Lysine → Arginine)	**Primer 1:** **5'**CGATGAAAACCTGAGGGCAGCCGACAGCG**3'** **Primer 2:** **5'**CGCTGTCGGCTGCCCTCAGGTTTTCATCG**3'**
pcDNA-E-cad/CTF2(RRRR)	pcDNA-E-cad/CTF2 (K740R,K818R) (Lysine → Arginine)	**Primer 1:** **5'**CGAGTGGGGCAACCGATTCAGGAGGCTGGCGGA CATGTACGGCGGTGGCG**3'** **Primer 2:** **5'**CGCCACCGCCGTACATGTCCGCCAGCCTCCTGA ATCGGTTGCCCCACTCG**3'**

Table 2.6 Details of constructs that were prepared by site-directed mutagenesis.

Table 2.7 describes constructs that were prepared by restriction digests of other constructs.

Construct	Insert (original construct)	Restriction Enzymes
pGEX-RFP	Full length of mouse Ret finger protein (pcDNA-FLAG-RFP)	NotI
pFA-CMV-ECad1	E-cadherin amino acids 734 – 822 (pBTM-ECad1)	EcoRI, PstI
pFA-CMV-ECad2	E-cadherin amino acids 734 – 884 (pBTM-ECad2)	EcoRI, PstI

Table 2.7 *Constructs prepared by restriction digests of other constructs. All fragments were cloned into indicated vectors that had been cut with the same enzymes, followed by alkaline phosphatase treatment.*

Table 2.8 describes siRNA oligos designed to knock-down p120 protein in human cells (ordered from Eurogentec).

Name of Oligo	Sequence
p120 1	**Sense:** r(CAGCAGAACUCCUCUUGGA)dTdT **Antisense:** r(UCCAAGAGGAGUUCUGCUG)dTdT
p120 2	**Sense:** r(CAGCAGUCAUUCAUAUGAU)dTdT **Antisense:** r(AUCAUAUGAAUGACUGCUG)dTdT
non-silencing control siRNA (AF 488)	**Purchased from Qiagen**

Table 2.8 *siRNA oligos used for transient transfections.*

2.1.12 DNA sequencing

All constructs prepared during this thesis were verified by automated DNA sequencing (MWG Biotech).

2.2 CELL BIOLOGY

2.2.1 Cell lines and culture conditions used

The cell lines human epithelial A431, human breast adenoma MCF-7, Madin-Darby canine kidney (MDCK), Human Embryonic Kidney (HEK) 293, and human colon adenocarcinoma SW620 were cultured in Dulbecco's Modified Eagle medium (DMEM, Gibco) supplemented with 10% complete fetal calf serum (FCS, PAA laboratories), 100U/ml penicillin/streptomycin (Gibco) and 1% GlutaMAX^{TM} (Gibco) (DMEM/10%FCS/pen/strep/glu). MDCK cells stably expressing E-cadherin-myc were kindly produced by Dr. S. (Dupre-Crochet et al., 2007), and cultured as above except that medium was supplemented with 800 μg/ml of G418 (Calbiochem). MDCK cells stably expressing GFP-RasV12 in tetracycline-inducible manner were kindly produced by Dr. Y. Fujita, and cultured as above except that tetracycline-free fetal calf serum was used (PAA laboratories) and medium was supplemented with 5 μg/ml of blasticidin (Invitrogen), and 400 μg/ml of Zeocin (Invitrogen). Cells were grown in incubators at 37°C in 5% CO_2.

2.2.2 Freezing and thawing cultured cells

To freeze cultured cells, aliquots containing $1x10^7$ cells were suspended in 1 ml of DMEM/10%FCS/pen/strep/glu supplemented with 10% DMSO and frozen at -80°C for 48 hrs before being stored permanently in liquid nitrogen.

To thaw cells, the aliquots were partially thawed at 37°C before being added to 10 ml of pre-warmed medium in a 15 ml tube. Cells were centrifuged at 200 x g for 5 min, resuspended in 5 ml of DMEM/10%FCS/pen/strep/glu, plated in 60 mm culture dishes and incubated overnight. Cells were then grown in fresh medium.

2.2.3 Passaging of A431, MCF-7, MDCK, HEK293 and SW620 cells

Cultured cells were passaged when they reached 80-90% confluency. Cells were washed using sterile phosphate-buffered saline (PBS) then treated with 1 ml

trypsin/EDTA (Gibco) per 90 mm culture dish for 2 min at 37°C. Dishes were gently tapped and the cells were resuspended in 9 ml of culture medium. Cells were counted using a haemocytometer and plated at the appropriate densities for experiments.

2.2.4 Transfection of HEK293 cells using Ca_2PO_4

$1x10^6$ cells per dish were plated in 90 mm dishes 24 hr before transfection so that that they were at a confluency of 30-50% at the time of transfection. A maximum of 15 μg of plasmid DNA per transfection was mixed with 450 μl dH_2O and 50 μl 2.5 M $CaCl_2$ and briefly centrifuged. The DNA mixture was added dropwise to 500 μl of 2x Hepes buffer (42 mM HEPES, 2 mM $Na_2HPO_4 \cdot 2H_2O$, 0.5 M NaCl, 20 mM KCl, pH 7.05, 0.22 μm filtered), vortexing after each dropwise addition. The solutions were left to stand for 5 min at RT and then added dropwise to the cells. Cells were incubated with the transfection solution for 4-6 hours before the medium was changed. 24 hrs after transfection, cells were used for examination of cell lysates, nuclear fractionation, reporter assays, immunoprecipitation and immunofluorescence experiments.

2.2.5 Transfection of MDCK, MCF-7, SW620 and COS-1 cells using Lipofectamine 2000™ reagent

Cells were plated at $2x10^6$ cells per dish in 90 mm dishes, or at $2x10^5$ cells per well in a 6 well plate so that they were 50-90% confluent at time of transfection. Cells were transfected 24 hours later with Lipofectamine 2000™ reagent (Invitrogen) according to the manufacturers instructions. Medium was changed 2 times after 3 hours. After a further 24 hours cells were used for examination of cell lysates, reporter assays and immunofluorescence experiments.

2.2.6 Transfection of HEK293 with siRNA using Hi-PerFect® reagent

5 nmol of lypholysed siRNA oligos from Qiagen were resuspended in 250 μl siRNA Suspension Buffer (Qiagen), resulting in a 20 μM solution. Samples were heated for 1 min at 90°C, then incubated at 37°C for 1 h, then stored at -20°C. Cells were plated in 24 well plates ($5x10^4$ cells per well) 24 hours before transfection. 1.5 μg of each siRNA oligo was mixed with 9 μl of Hi-PerFect®

reagent (Qiagen) in 100 µl serum free, antibiotic free DMEM. The resulting solution was incubated at RT for 15 min, and then added dropwise to the cells. 24 hours after siRNA transfection, cells were then transfected with E-cad/CTF2. After a further 48 hours, cells were either lysed in Triton-X-100 lysis buffer or used for nuclear fractionation. Table 2.6 describes siRNA oligos that were used in this thesis.

2.2.7 Establishment of MDCK cells stably expressing GFP-E-cad/CTF2-NLS in a tetracycline-inducible manner.

MDCK cells previously transfected with pcDNA6/TR using Lipofectamine 2000, and selected in medium containing 5 µg ml^{-1} of blasticidin (Invitrogen) were transfected again with pcDNA4/TO/GFP-E-cad/CTF2-NLS using Lipofectamine 2000. The doubly transfected cells were selected in medium containing 10 % tetracycline-free FCS (PAA, UK), 5 µg ml^{-1} of blasticidin, and 400 µg ml^{-1} of Zeocin (Invitrogen). For selection, cells were split 24 hours after transfection at dilutions of 1:5, 1:20, 1:150 and 1:500 (2 plates were used per dilution). After a further 24 hours, selection antibiotics were applied. After 1-2 weeks, colonies were picked up using filter papers soaked in trypsin.

2.2.8 Reagents used in cell biology experiments

Table 2.9 gives details of the primary antibodies that were used in this thesis. Concentrations of the stock solutions and working dilutions used for immunofluorescence and western blotting are shown.

Primary Antibody	Stock Conc.	Working dilution (Western blotting)	Working dilution (Immunofluorescence)
Mouse anti-E-cadherin (BD Transduction Laboratories)	0.25 mg/ml	1:3000	-
Mouse anti-FLAG, Clone M2 (Sigma)	4.9 mg/ml	-	1:100
Mouse anti-FLAG, Clone M2 POD (Sigma)	1 mg/ml	1:5000	-
Mouse anti-Myc clone 4A6 (Upstate)	0.3 mg/ml	1:2000	1:100
Rat anti-HA Clone 3F10 (Roche)	0.2 mg/ml	-	1:100
Mouse anti-pp120 (BD Biosciences)	0.25 mg/ml	1:1000	-
Mouse anti-N-cadherin (BD Transduction Laboratories)	0.25 mg/ml	1:1000	-
Mouse anti-GM130 (BD Transduction Laboratories)	0.25 mg/ml	1:250	-
Mouse anti-BiP/GRP78 (BD Transduction Laboratories)	0.25 mg/ml	1:250	-
Mouse anti-Lamp-1 (BD Transduction Laboratories)	0.25 mg/ml	1:250	-
Rat anti-tubulin (Abcam)	1.01 mg/ml	1:1000	-
Rabbit anti-CBP (Santa Cruz)	200 µg/ml	1:1000	-
Rabbit anti-RFP (IBL)	0.1 mg/ml	1:1000	-
Mouse anti-Ubiquitin (Zymed)	0.5mg/ml	1:500	-
Rabbit anti-SUMO (Abgent)	0.25 mg/ml	1:500	-

Table 2.9 *Details of primary antibodies used in Western blotting and immunofluorescence experiments.*

Table 2.10 describes the inhibitors that were used during this research. Details of the stock solutions and treatment conditions are provided.

Inhibitor	Function	Details
MG132 (Sigma)	Proteasome (20S and 26S subunits) inhibitor.	4 µl of 50 mM stock (20 µM final conc) added to 10 ml DMEM culture medium and incubated for 6 hr.
γ-secretase inhibitor X (Calbiochem)	Inhibitor of γ-secretase	Added to DMEM culture medium at 12.5 nM for 6 hr.
Staurosporine (Upstate)	Inhibitor of protein kinase C and other protein kinases (potent inducer of apoptosis)	Added to DMEM culture medium at 1 µM for 6 hr.
IC261 (Calbiochem)	Inhibitor of Casein Kinase 1	Added to DMEM culture medium at 5 µg/ml for 4 hr.
Okadaic Acid (Calbiochem)	Inhibitor of protein phosphatases PP1 and PP2A	Added to DMEM culture medium at 3.13 ng/ml for 4 hr.

Table 2.10 *Details of inhibitors used during this research, including their functions and the conditions for treatments.*

Table 2.11 describes other treatments used during this research.

Treatment	Function	Details
Hepatocyte Growth Factor (HGF) Human Recombinant (Chemicon)	Activates the tyrosine kinase receptor, c-Met.	Added to DMEM culture medium at 50 ng/ml and incubated for 24 hr.
Ultra Violet (UV)	Induction of apoptosis	2X or 3X auto-crosslink (1600) using UV stratalinker 1800 (Stratagene) followed by incubation for 6 hr
λ-phosphatase (New England Biolabs)	De-phosphorylation of proteins and nucleic acid.	100 units added to immunoprecipitated proteins for 30 minutes at 30 °C

Table 2.11 *Details of other treatments used during this research, including their functions and the conditions used.*

2.2.9 Immunofluorescence microscopy

Cells were plated onto glass coverslips (13 mm, No. 1½, BDH) at densities as described in sections 2.2.4 and 2.2.5, and transfected. After 24 hours, cells were washed twice with PBS, then fixed in 1 ml 3% paraformaldehyde-PBS for 15 min at RT. After washing with PBS, cells were permeabilised in 0.5% Triton X-100-PBS for 15 min, then blocked with DMEM containing 10% FCS for 1 hr.

Primary antibody, diluted in DMEM/10% FCS as detailed in table 2.9 was added for 1 hour before washing 5 times with PBS, incubated. Cytochrome 2 (Cy2) or Rhodamine-Red-X-conjugated secondary antibodies (Jackson Immunochemicals) were added at 1:100 in DMEM/10% FCS for 1 hour, and washed 5 times with PBS. Hoechst solution (1:5000 dilution, Molecular Probes) was added to coverslips for approximately 1 min and washed 5 times with PBS. The coverslips were then mounted onto glass slides using Mowiol (5 μl/coverslip, Calbiochem). To obtain epifluorescence images, a Zeiss Axipoplan2 microscope using a 40x 1.3 oil immersion objective was used at room temperature (Zeiss). Images were captured using a C4742-95 digital camera (Hamamatsu) and Openlab software (Improvision). Confocal images were obtained with a Bio-Rad confocal mounted on a Nikon Optiphot 2 microscope using a 60x 1.4 oil-immersion objective at room temperature, or a Leica SPE confocal microscope with a 63x 1.3 oil-immersion objective at room temperature. Images were aquired using Laser Sharp software (Bio-Rad) or Leica Application Suite, respectively. Merged images were split using Image J software (National Institute of Health). The ratio of nucleus/cytoplasm fluorescence intensity in confocal images were analysed by measuring fluorescence intensity in a region (diameter 1.97 μm) using Image J. Brightness and contrast were adjusted using Photoshop CS (Adobe).

2.2.10 Phase construct microscopy

To obtain phase-contrast images, a Leica DM IRB microscope using a 10x 0.25 air objective was used at room temperature (Leica). Images were captured using an Orca camera (Hamamatsu) and Openlab software (Improvision).

2.3 PROTEIN BIOCHEMISTRY

2.3.1 Preparation of cell lysates

Cultured cells were scraped in 1 ml of cold PBS, and centrifuged at 400 x g for 3 min at 4°C. Pelleted cells were then lysed in 1 ml of cold lysis buffer (20 mM Tris/Cl pH 7.5, 150 mM NaCl, 1% (v/v) Triton X-100) supplemented with 5 μg ml^{-1} leupeptin (Sigma), 50 mM PMSF, 7.2 trypsin inhibitor units ml^{-1} of aprotinin (Sigma), and 1.25 mg/ml N-ethylmaleimide before being vortexed briefly. 50% of the sample volume of SDS loading buffer was added and the resulting solution was heated at 95°C.

SDS-PAGE loading buffer was prepared as follows: 2.7g of SDS was added to 4.5 ml of glycerol, 11.2 ml of 0.5 M Tris/Cl (pH 6.5) and the solution was made up to 28.2 ml with dH_20. Bromophenol blue was added to a final concentration of 1mg/100 ml. 14M of 2-mercaptoethanol was added at a ratio of 94:6 (buffer:2-mercaptoethanol).

2.3.2 Nuclear fractionations

Cells were washed twice with PBS and trypsinized until well separated and centrifuged at 200 x g for 5 min. Pelleted cells were resuspended in 150 μl 2X lysis buffer (50 mM Hepes/NaOH pH 7.4, 10 mM EGTA, 5 mM $MgCl_2$, 20% Glycerol, and 2% NP-40) containing 5 μg ml^{-1} leupeptin, 50 mM PMSF, 7.2 trypsin inhibitor units ml^{-1} of aprotinin, and 10 mM *N*-ethylmaleimide. The resuspension was then triturated with a 25G needle 12 times. After centrifugation at 110 X g for 5 min at 4°C, the supernatant was removed and the pelleted nuclei were washed twice in 1X lysis buffer. The nuclear fractions were then boiled for 10 min with SDS-PAGE loading buffer, followed by Western blotting, or lysed in 1 ml of Triton X-100 lysis buffer followed by immunoprecipitation. Purity of nuclear fractions was confirmed by Western blotting with antibodies against several compartment-specific markers (Figure 4.7).

2.3.3 Immunoprecipitations

Cells were scraped in 1 ml ice-cold PBS, and centrifuged at 400 x g for 3 min at 4°C, then lysed as described in section 2.3.1. Lysates were rotated for 30 min at 4°C, then centrifuged at 5000 x g, then 100 µl aliquots were removed in order to later examine the input. 800 µl of the remaning supernatants were added to microfuge tubes containing 100 µl of a 50% slurry of protein G-Sepharose^{TM} 4B (Amersham) conjugated to the antibody used for immunoprecipitation (prepared by coupling 30 µl of Protein G beads with 5 µl of antibody per reaction in a total volume of 500 µl of lysis buffer at 4°C with rotation). Supernatants were rotated with antibody beads for 1 hr at 4°C, and centrifuged at 5000 x g for 1 min at 4°C. Beads were washed twice in lysis buffer and then resuspended in SDS-PAGE loading buffer. Samples were boiled for 10 min at 95°C.

2.3.4 Preparation of GST-RFP

BL21 *E.coli* were transformed with pGEX-RFP and spread onto agar plates containing 50 µg/ml ampicillin. 3 ml of LB containing 50 µg/ml ampicillin (LB-amp) was inoculated with a single colony and grown for four hours and then added to 200 ml of LB-amp, which was grown overnight at 37°C with agitation. 20 ml of the overnight culture was used to inoculate 750 ml of LB-amp and grown at 37°C with agitation until the OD_{600} reading was between 0.3-0.4. Production of GST-RFP was induced by addition of 750 µl of isopropyl-B-D-thiogalactopyranoside (IPTG, Melford Laboratories Ltd) to the 750 ml culture. Cultures were incubated for 4 hours at 30°C with gentle agitation and the cells were then centrifuged at 3400 x g for 20 min. The cells were washed once with 40 ml PBS and pellets were stored at -80°C.

Bacterial pellets were thawed on ice, resuspended in 20 ml lysis buffer (20 mM Tris/Cl pH7.5, 10% (w/v) Sucrose (Sigma), 1 mM Dithiothreitol (DTT, Sigma), 1% Triton X-100, Sigma) or in RIPA buffer (150 mM NaCl, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS, 50 mM Tris/Cl (pH 8.0)), and supplemented with 1 mM phenyl methyl sulphonyl fluoride (PMSF, Sigma). Resuspended bacterial cells were sonicated on ice (amplitude 5 microns) five times for 30 sec

each, with a 1 min interval between each pulse, using a Soniprep 150 (Sanyo). The lysate was centrifuged at 50,000 x g for 1 hr at 4°C. The supernatant was carefully removed and filtered through a 0.45 μm filter and then 1 ml was added to 30 μl of glutathione-Sepharose™ 4B beads (Amersham). The suspension was rotated for 1 hr at 4°C, beads were washed before additon of SDS buffer for analysis by SDS PAGE.

GST-RFP was isolated from Inclusion Bodies using the Protein Refolding Kit (Novagen). Briefly, cells were thawed on ice and resuspended in 75 ml of 1X Wash Buffer (20 mM Tris-HCl pH 7.5, 10 mM EDTA, 1% Triton X-100). Lysozyme was added to a final concentration of 100 μg/ml from a freshly prepared 10 mg/ml stock in water and the suspension was incubated at 30°C for 15 min, then sonicated on ice (amplitude 5 microns) in five 30 sec bursts, with a 1 min interval between each pulse, using a Soniprep 150 (Sanyo). Immediately following sonication, 100 mM phenyl methyl sulphonyl fluoride (PMSF, Sigma) was added and the inclusion bodies were collected by centrifugation at 10,000 x g for 10 min. The supernatant was removed and the pellet was thoroughly resuspended in 75 ml of 1X IB Wash Buffer, before centrifugation again at 10,000 x g for 10 min. The pellet was resuspended again in 75 ml of 1 X IB Wash Buffer. The suspension was transferred to a clean centrifuge tube of known weight. Inclusion bodies were collected by centrifugation at 10,000 x g for 10 min. The supernatant was decanted and the last traces were removed by tapping the inverted tube on a paper towel. The weight of inclusion bodies was determined and inclusion bodies were thoroughly resuspended in 1X IB Solubilsation Buffer supplemented with 0.3% N-lauroylsarcosine and 1 mM DTT to a concentration of 10-20 mg/ml. The suspension was incubated at room temperature for 15 min and clarified by centrifugation at 10,000 x g for 10 min at room temperature. The supernatant containing solubilised protein was transferred to a clean tube, avoiding pelleted debris. To allow protein refolding, the sample was dialysed for 3 h at 4°C using a volume of 1X Dialysis Buffer (supplemented with 0.1 mM DTT), which was 50X that of the solublised protein sample. 1X Dialysis Buffer was changed and dialysis was continued overnight. Dialysis was continued for two additional changes (3 hours each) using 1X Dialysis Buffer

without DTT. The dialysed protein solution was centrifuged at 10,000 x g for 10 min at 4°C. The solution was then aliquoted and stored at -80°C. Protein concentration was determined by SDS-PAGE with BSA standards.

2.3.5 GST pull-down assays

Cells were scraped in 1 ml ice-cold PBS, and centrifuged at 400 x g for 3 min at 4°C, then lysed as described in section 2.3.1. Lysates were rotated for 30 min at 4°C, then centrifuged at 5000 x g, then 100 µl aliquots were removed in order to later examine the input. 800 µl of the remaning supernatants were added to microfuge tubes containing 100 µl of a 50% slurry of glutathione-Sepharose™ 4B conjugated to GST alone or the GST-RFP (prepared by coupling 30 µl of glutathione-Sepharose™ beads with 20 µg of GST/GST-RFP per reaction in a total volume of 500 µl of lysis buffer at 4°C with rotation). Supernatants were rotated with glutathione beads for 1 hr at 4°C, and centrifuged at 5000 x g for 1 min at 4°C. Beads were washed twice in lysis buffer and then resuspended in SDS-PAGE loading buffer. Samples were boiled for 10 min at 95°C.

2.3.6 Nickel precipitation of HIS-tagged proteins

Cells were scraped in 1 ml ice-cold PBS, and centrifuged at 400 x g for 3 min at 4°C, then lysed as described in section 2.3.1. Lysates were rotated for 30 min at 4°C, then centrifuged at 5000 x g, then 100 µl aliquots were removed in order to later examine the input. 800 µl of the remaning supernatants were added to microfuge tubes containing 100 µl of a 50% slurry of Ni-NTA Agarose (Qiagen) (30 µl used for each precipitation, centrifuged and washed with lysis buffer). Supernatants were rotated with Ni-NTA Agarose for 1 hr at 4°C, and then centrifuged at 5000 x g for 1 min at 4°C. Beads were washed twice in lysis buffer and then proteins were eluted from the beads using 150 µl of 250 mM Imidazole in PBS, by shaking for 30 min at 4°C. SDS-PAGE sample buffer was added and samples were boiled for 10 min at 95°C.

2.3.7 DNA-Cellulose binding assays

Cells were lysed in 750 µl buffer containing 10 mM Hepes/NaOH pH 7.4, 0.1% Triton X-100, 100 mM NaCl, 10% glycerol, and 0.05 mM EDTA supplemented with 5 µg ml^{-1} leupeptin, 50 mM PMSF, 7.2 trypsin inhibitor units ml^{-1} of aprotinin, and 10 mM *N*-ethylmaleimide. Lysates were sonicated on ice (amplitude 4 microns) in three 15 sec bursts, with a 30 sec interval between each pulse, using a Soniprep 150 (Sanyo), and clarified by centrifugation at 5000 x g for 20 min. 50 µl of cell lysate or purified protein (75 ng) was incubated with 25 µl DNA-cellulose (GE Healthcare) or cellulose (Sigmacell cellulose type 50; Sigma) in 100 µl of lysis buffer for 1 h at 4°C. For competition assays, cell lysates were pre-incubated with the indicated amounts of DNA (sonicated, calf thymus; GE Healthcare) or RNA (calf-liver type IV; Sigma) and rotated for 30 min at 4°C, and then added to DNA-cellulose beads. After rotation, the beads were washed four times with 1 ml lysis buffer with vortexing, and the amounts of protein retained on the beads were determined by Western blotting.

2.3.8 Protein concentration quantification

To ensure equal loading in SDS-PAGE, the protein concentration of lysates was quantitated using the D_C Protein Assay reagent (Bio-Rad) and measured on a VERSAmax microplate reader (Molecular Devices).

2.3.9 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gels were prepared as described in Table 2.12, using reagents from Sigma.

Reagent	10%	10% (mini)	12%	12% (mini)	Stacking	Stacking (mini)
dH_2O	12 ml	3 ml	10 ml	2.5 ml	9.5 ml	3.2 ml
1.5 M Tris/Cl pH 8.8	10 ml	2.5 ml	10 ml	2.5 ml	-	-
0.5 M Tris/Cl pH 6.8	-	-	-	-	3.75 ml	1.25 ml
50% Glycerol	8 ml	2 ml	8 ml	2 ml	-	-
40% Acrylamide	10 ml	2.5 ml	12 ml	3 ml	1.5 ml	500 µl
10% SDS	400 µl	100 µl	400 µl	100 µl	150 µl	50 µl
TEMED	40 µl	10 µl	40 µl	10 µl	15 µl	5 µl
10% APS	100 µl	25 µl	100 µl	25 µl	150 µl	50 µl

Table 2.12 *Constituents of polyacrylamide running and stacking gels.*

Protein samples were mixed with SDS loading buffer and heated to 95°C for 10 min before loading. Gels were run in Bio-Rad Protean II™ or Mini Protean II™ tanks using Laemmli buffer (National Diagnostics). Protean II gels were run at 9 mA (one gel) or 18 mA (two gels) overnight at RT or at 35 mA (one gel) or 70 mA (two gels) in four hours. Mini protean II gels were run at 25 mA for 1 hour. ColorBusrt™ marker (Sigma) was loaded as a molecular weight marker.

2.3.10 Coomassie staining of polyacrylamide gels

Coomassie stain (0.04% w/v Brilliant blue R, 5% v/v methanol and 5% v/v glacial acetic acid) was added to polyacrylamide gels and gently rocked for 1 hr. Gels were destained using several changes of polyacrylamide gel destain (40% methanol and 10% glacial acetic acid) until the protein bands could be clearly detected. Gels were dried onto Whatmann 3M filter paper using a heated, vacuum-assisted gel drier (Bio-Rad).

2.3.11 Western blotting

Polyvinylidene difluoride (PVDF, Millipore) membranes were soaked in methanol and then shaken in transfer buffer (25 mM Tris, 192 mM glycine and 10% methanol) for at least 10 minutes. The gel to be transferred, sponges and 6 pieces of Whatmann 3M filter paper were also soaked in transfer buffer for at least 10 minutes. Proteins from the gel were transferred to the membrane in a Bio-Rad Trans-Blot® electrophoretic transfer cell filled with transfer buffer. 3 filter papers and a sponge were placed on either side of the gel and membrane. Transfer was performed either overnight at 220 mA at 4°C, or for 2 hrs at 70 V at 4°C.

Membranes were blocked with shaking for 1 hour with 3% 'Marvel' milk powder-PBS. Primary antibody or peroxidase-conjugated anti-FLAG antibodies were added, as detailed in table 2.9 for either 1 hr at RT or overnight at 4°C with shaking. For peroxidase-conjugated anti-FLAG antibody, membranes were washed 5 times for 10 min at RT in PBS-Tween® (0.05% v/v Tween® 20, Sigma). For unconjugated primary antibodies, membranes were washed 3 times for 10 min each at RT in PBS-Tween® and then peroxidase conjugated secondary antibodies (Jackson Immunochemicals) were added in 3% milk/PBS for 1 hr at RT with shaking. Secondary antibodies were washed using 5 x 10 min washes in PBS-Tween®. Bands on the membrane were visualised using ECL western blotting detection reagents (Amersham) or as prepared in table 2.13 (equal volumes of the two reagents were mixed and the membrane was incubated for 2 min) and high performance chemiluminescence film (Hyperfilm, Amersham) according to the manufacturers instructions.

Reagent	Enhanced Luminol Reagent	Oxidising Reagent
250 mM Luminol	1 ml	-
90 mM p-coumaric acid	0.5 ml	-
1 M Tris/Cl pH 8.5	10 ml	10 ml
30% Hydrogen peroxide	-	70 µl
Total volume (make up with autoclaved water)	100 ml	100 ml

Table 2.13 *Constituents of ECL reagents.*

2.4 YEAST TRANSACTIVATION ASSAYS

2.4.1 Production of yeast competent cells

Cells from a single colony from a YPD plate were inoculated into 20 ml of YPD medium, and incubated at 30°C overnight, starting in the morning. 20 ml of the overnight culture were transferred to 300 ml of YPD medium in a one-litre flask, and incubated at 30°C until the OD_{600} reached 0.4-0.5 (2-4 hours). Cells were centrifuged at 1000 X g for 10 min at room temperature. The supernatant was discarded and the pellet was suspended with 6 ml of LiAc/TE before being transferred to a 15 ml tube and centrifuged at 2300 rpm at room temperature. The supernatant was discarded and the pellet suspended with 2 ml of LiAc/TE.

YPD medium was produced using 20 g/l Difco peptone, 10 g/l Yeast extract, and 15-20 g/l agar (for plates only). pH was adjusted to 5.8 and the solution was autoclaved. Glucose was added to 2% by adding 50 ml of a 40% stock solution (separately autoclaved) to each litre of media.
LiAc/TE solution contains 1 ml of 10 X LiAc (1 M lithium acetate (Sigma) adjusted to pH 7.5 with diluted acetic acid and autoclaved); 1 ml of 10 X TE (0.1 M Tris-HCl, 10 mM EDTA, pH 7.5 autoclaved) and 8 ml of autoclaved H_20.

2.4.2 Production of –T selection plates

-T selection plates were prepared using 3.35 g of yeast nitrogen base (without amino acid) and 8 g of agar in 425 ml of water. The solution was autoclaved and kept at 50 – 55°C. Prewarmed, autoclaved solutions were then added as follows: 25 ml 40% glucose; 50 ml 10 X amino acid solution (300 mg/l L-Isoleucine; 1500 mg/l L-Valine; 200 mg/l L-Adenine hemisulfate salt; 200 mg/l L-Arginine HCl; 200 mg/l L-Histidine HCl monohydrate; 1000 mg/l L-Leucine; 300 mg/l L-lysine HCl; 200 mg/ml L-Methionine; 500 mg/l L-Phenylalanine; 2000 mg/ml L-threonine; 300 mg/l L-Tyrosine; and 200 mg/l L-Uracil (all from Sigma); note that 200 mg/ml L-Tryptophan was omitted for selection purposes); and 10 ml of

1M 3-aminotriazole (3-AT) (Sigma), filter sterilised. The solution was mixed well and poured onto square plates (25-30 ml per plate).

2.4.3 Transformation into yeast

50 µl yeast cells were mixed with 2.5 µl salmon sperm DNA and 2 µl of plasmid to be transformed (if from maxi prep; 5 µl if from mini prep). 300 µl of freshly prepared PEG/LiAc solution (8 ml of 50% polyethylene glycol (PEG); 1 ml of ml of 10X TE; 1 ml of 10X LiAc) was added and vortexed to mix, before incubating at 30°C for 30 min with shaking. 35 µl of DMSO was added and vortexed to mix. Cells were heat shocked for 15 min in a 42°C waterbath, then chilled on ice and centrifuged for 5 sec at 14000rpm. The supernatant was removed and the cells were resuspended in 0.5 ml of TE. Cells were then plated on the –T selection plates.

2.4.4 Filter assay for β-galactosidase

This assay was used when the colonies were at least 1-2 mm in diameter. Z buffer was prepared as follows: 16.1 g/l $Na_2HPO_4.7H_20$; 5.5 g/l $NaH_2PO_4.H_20$; 0.75 g/l KCL; 0.246 g/l $MgSO_4.7H_20$; pH 7.0). X-gal stock solution was prepared by dissolving 5-bromo-4-chloro-3-indolyl-β--D-galactopyranoside (X-gal) in N,N-dimethylformamide (DMF) at a concentration of 20 mg/ml and stored in the dark at -20°C. Sterile Whatman #1 filters were presoaked in Z buffer/X-gal solution (100 ml of Z buffer; 0.27 ml of β-mercaptoethanol; 1.67 ml of X-gal stock solution) as follows: 5 ml of Z buffer/X-gal solution was added to a clean 150-mm plate and a 125-mm filter was layered onto the liquid to soak it up. A sterile Whatman #1 filter was placed over the surface of the plate containing the transformant colonies. Holes were poked through the filter into the agar to orient the filter on the agar. The filter was carefully lifted off the agar plate with forceps and transferred (colonies facing up) to a pool of liquid nitrogen and submerged for 10 sec. After the filter had completely frozen, it was removed and allowed to thaw at room temperature. The filter was placed colony side up on another filter that was presoaked in Z buffer/X-gal solution as before,

avoiding trapping air bubbles under the filter. The filters were incubated at 30°C or room temperature and checked periodically for the appearance of blue colonies.

2.4.5 Liquid culture assay for β-galactosidase

Individual yeast transformant colonies were inoculated into synthetic selection media (-T) containing glucose (as for selection plates without agar) and incubated at 30°C until the culture reached mid-log phase ($OD_{600} \approx 1.0$). For each culture, 0.1 ml of culture and 0.7 ml of Z buffer containing β-mercaptoethanol (0.27 ml of β-mercaptoethanol in 100 ml of Z buffer) were added to a 1.5 ml microfuge tube. 50 µl of $CHCl_3$ and 50 µl of 0.1% SDS were added and the solution was vortexed for 30 sec. 0.16 ml of o-nitophenylgalactoside (ONPG) (Sigma) solution (4 mg/ml in 0,1 M phosphate buffer, pH 7.0) was added and incubated at 30°C for 1 hour before quenching the reactions with the addition of 0.4 ml of 1 M Na_2CO_3 and centrifuging for 10 min at 14,000 rpm to remove cell debris. Absorbance was read at 420 nm and β-galactosidase activity was measured using the following equation: β-galactosidase units = 1000 x [OD_{600} /t x V x OD_{600}] where t = time (min) of incubation and V = volume (ml) of culture added to Z buffer.

2.5 MAMMALIAN REPORTER ASSAYS

2.5.1 Mammalian transactivation assays.

HEK293 cells were seeded at 1 x 10^6 in a 9 cm plate and transfected with pFR-luciferase (firefly) and pCMV-Renilla (50X less than pFR-luciferase (firefly) together with pFA-CMV-ECad1 and or pFA-CMV-ECad2. After 24 hours cells were split into a 48 well plate (5 x 10^4 per well). After a further 24 hours, Firefly and Renilla luciferase activities were measured using the dual luciferase assay kit (Promega, Madison, WI) and a Turner Designs TD-20/20 luminometer, according to the manufacturer's instructions.

2.5.2 Reporter assays using TOP-FLASH or KBS reporter constructs

SW620 cells were transfected with pTOP-FLASH, pFOP-FLASH, pGL3-myc or pGL3-cyclin D1 together with E-cad/CTF2 or E-cad/CTF2-NLS-myc. HEK293 and cells were transfected with pGL3-control vector or pGL3-4xKBS together with FLAG-p120, E-cad/CTF2, E-cad/CTF2-NLS-myc, or E-cad/CTF2(AAA)-NLS-myc. For reporter assays using the *matrilysin* promoter, HEK293 cells were transfected with pGL3-Basic-2.3kb HMat together with FLAG-p120, E-cad/CTF2-NLS-myc, or E-cad/CTF2(AAA)-NLS-myc. LacZ was used as a control for transfection efficiency in all experiments. Luciferase activity was measured using the luciferase assay kit according to the manufacturer's instructions (Promega, Madison, WI) and a Turner Designs TD-20/20 luminometer. β-Gal activity was measured using the β-galactosidase assay kit according to the manufacturer's instructions (Promega) and the VERSAmax microplate reader (Molecular Devices).

2.6 COMPUTATIONAL ANALYSIS

All image analysis was performed using Adobe Photoshop, version 6.0. Quantitative data was depicted using Microsoft Excel and Quantity One software. Sequence alignments were performed using Clustal W software. Student's *t* tests assuming equal or unequal variance and a two-tailed distribution were performed for statistical analysis using Microsoft Excel.

CHAPTER 3

3 ANALYSIS OF STIMULI INDUCING PRODUCTION OF THE E-CADHERIN CLEAVAGE PRODUCT, E-CAD/CTF2

3.1 Introduction

As described in section 1.4.5 (Introduction), multiple proteases are known to cleave E-cadherin. Various extracellular metalloproteases cleave E-cadherin, removing most of the extracellular domain (Lochter et al., 1997; Maretzky et al., 2005; Noe et al., 2001). The role of the released extracellular domain as an invasion promotor has been well characterised (Lochter et al., 1997; Noe et al., 2001), but the fate of the remaining fragment, E-cad/CTF1, has not been elucidated. E-Cad/CTF1 has recently been shown to be cleaved by γ-secretase, in response to treatment with staurosporine or the *Bacteroides fragilis* toxin, releasing E-cad/CTF2 (Marambaud et al., 2002; Wu et al., 2007). This cleavage by γ-secretase is of particular interest from a signalling perspective, since many intracellular domain fragments of transmembrane proteins released by γ-secretase-mediated cleavage localise to the nucleus and have signalling functions (Section 1.5.3, Introduction) (Fortini, 2002). However the physiological stimuli inducing γ-secretase-mediated cleavage of E-cadherin have not been addressed. In this chapter, various stimuli that influence the cleavage of E-cadherin to produce E-cad/CTF2 were investigated.

3.2 Characterisation of cleavage products of E-cadherin in epithelial cells

3.2.1 Domain structure of E-cadherin cleavage products

As described in sections 3.1 and 1.4.5 (Introduction), various intracellular cleavage products are released following cleavage by different proteases. E-Cad/CTF1 is the 38 kDa cleavage product induced by extracellular metalloproteases that cleave close to the interface between the extracellular and transmembrane regions of E-cadherin (Lochter et al., 1997; Maretzky et al.,

2005; Noe et al., 2001). E-Cad/CTF2 (33 kDa) is the result of γ-secretase cleavage, which occurs following removal of most of the extracelluar region by metalloproteases (Marambaud et al., 2002; Wu et al., 2007). E-Cad/CTF3 (29 kDa) results from cleavage by caspase-3 after induction of apoptosis (Keller and Nigam, 2003; Marambaud et al., 2002; Steinhusen et al., 2001). The size and domain structure of these intracellular cleavage products are shown in Figure 3.1

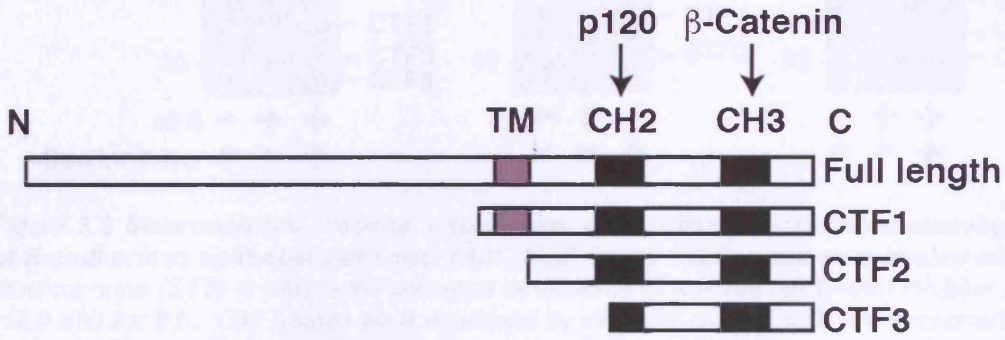


Figure 3.1 ***Schematics for the domain structure of full length E-cadherin, E-cad/CTF1, E-cad/CTF2, and E-cad/CTF3.*** *The intracellular region of E-cadherin contains two CH (cadherin homology) domains, CH2 and CH3, which bind to p120 and β-catenin, respectively. TM; transmembrane region.*

3.2.2 Staurosporine induces γ-secretase-mediated and caspase-3-mediated cleavage of E-cadherin in epithelial cell lines

First, the proteolytic cleavage of E-cadherin was examined in several epithelial cell lines. As previously reported (Marambaud et al., 2002), treatment of the human epithelial cell line A431 with staurosporine lead to the production of cytoplasmic cleavage products, E-cad/CTF2 and E-cad/CTF3 (Figure 3.2). Staurosporine is a potent inhibitor of protein kinase C (PKC) and other protein kinases, which strongly induces apoptosis (Tamaoki et al., 1986). E-Cad/CTF2 was also observed in the human breast adenocarcinoma cell line MCF-7 and Madin-Darby canine kidney (MDCK) cells after treatment with staurosporine (Figure 3.2). E-Cad/CTF1 was also present in all three cell lines (Figure 3.2). The identity of a number of other bands between 45 and 100 kDa, particularly in MDCK cells, is not known and varies between different experiments. As expected, it was observed that treatment with γ-secretase inhibitor X specifically

blocked staurosporine-induced production of E-cad/CTF2 in all three cell lines and lead to an accumulation of its precursor E-cad/CTF1 (Figure 3.2).

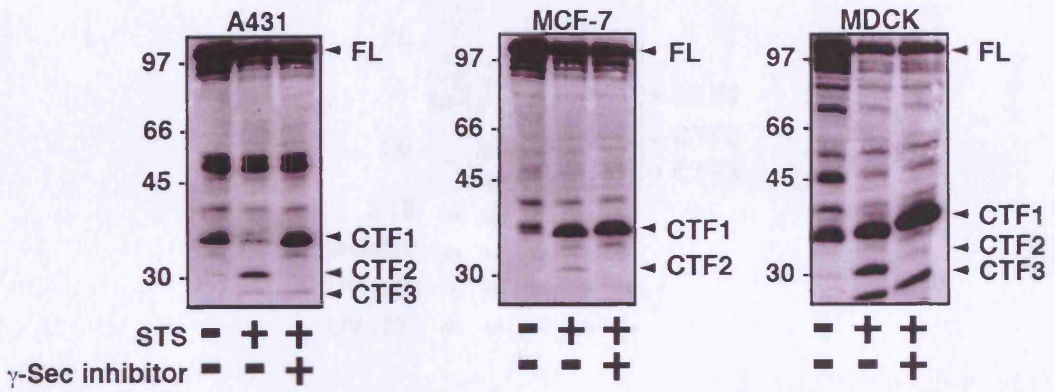


Figure 3.2 ***Staurosporine induces γ-secretase- and caspase-3-mediated cleavage of E-cadherin in epithelial cell lines.*** *A431, MCF-7, and MDCK cells were treated with staurosporine (STS) (1 μM) in the presence or absence of γ-secretase (γ-sec) inhibitor X (12.5 nM) for 6 h. Cell lysates were examined by Western blotting with anti-E-cadherin antibody. The positions of full length E-cadherin (FL) and the C-terminal fragments E-cad/CTF1, E-cad/CTF2, and E-cad/CTF3 are indicated with arrowheads.*

3.3 Analysis of stimuli that induce the production of E-cad/CTF2

3.3.1 Apoptosis induces production of E-cad/CTF2 in A431 cells

The data in Figure 3.2 indicate that staurosporine induces γ-secretase-mediated cleavage of E-cadherin. Staurosporine induces apoptosis which is indicated by the production of the caspase-3 cleavage product of E-cadherin, E-cad/CTF3 (Figure 3.2). However it is also a potent inhibitor of protein kinase C and other protein kinases. Thus it is difficult to conclude from this data alone whether the induction of apoptosis itself stimulates γ-secretase-mediated cleavage of E-cadherin. To further investigate this possibility, A431 cells were exposed to Ultra-Violet light (UV) in order to induce apoptosis. Under these conditions the cells showed apoptotic cellular phenotypes of rounding-up, blebbing and an increase in cells detached from the plate compared to controls, as observed by phase contrast microscopy. Upon exposure to UV, Western blot bands of the same molecular size as E-cad/CTF2 and E-cad/CTF3 were produced, which were also observed upon treatment with staurosporine (Figure 3.3).

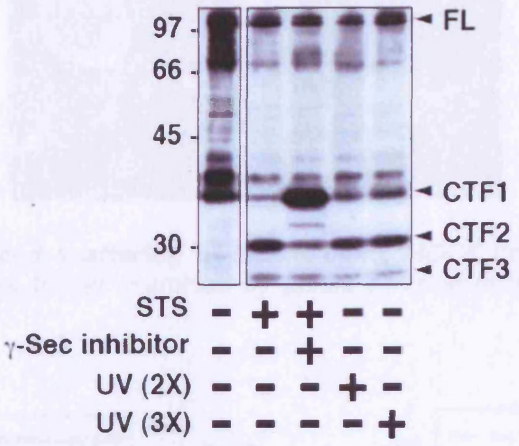


Figure 3.3 ***UV treatment induces production of E-cad/CTF2 and E-cad/CTF3 in A431 cells.*** *A431 cells were treated with staurosporine (STS) (1 μM) in the presence or absence of γ-secretase (γ-sec) inhibitor X (12.5 nM) for 6 h or with UV as detailed in Table 2.9 (Chapter 2). Cell lysates were examined by Western blotting with anti-E-cadherin antibody. The positions of full length E-cadherin (FL) and the C-terminal fragments E-cad/CTF1, E-cad/CTF2, and E-cad/CTF3 are indicated with arrowheads. Images shown for the first lane and the other four lanes are from the same film.*

3.3.2 Hepatocyte Growth Factor (HGF) induces γ-secretase-mediated cleavage of E-cadherin in MDCK cells.

Next, E-cadherin cleavage in epithelial cells in response to another stimulus that leads to the loss of cell-cell contacts was investigated. As described in section 1.4.1 (Introduction), treatment of MDCK cells with hepatocyte growth factor (HGF) activates the tyrosine kinase HGF receptor (c-Met) and induces endocytosis of E-cadherin, leading to the disruption of cell-cell contacts and a fibroblast-like morphological change (Fujita et al., 2002; Kamei et al., 1999; Weidner et al., 1990) (Figure 3.4). The effect of HGF treatment on the proteolytic cleavage of E-cadherin was examined. A 33 kDa fragment of the same size as E-cad/CTF2 was induced when MDCK cells were treated with HGF (Figure 3.5, left). Induction of E-cad/CTF2 by HGF treatment was blocked by addition of γ-secretase inhibitor X (Figure 3.5, right). These data indicate that E-cad/CTF2 is produced by γ-secretase cleavage when intercellular adhesions are disrupted in epithelial cells by activation of the HGF receptor.

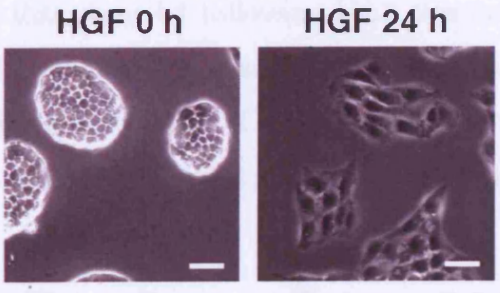


Figure 3.4 ***HGF-induced scattering of MDCK cells.*** *MDCK cells were treated with HGF (50 ng/ml) for 24 h and examined by phase-contrast microscopy. Scale bars represent 50 μm.*

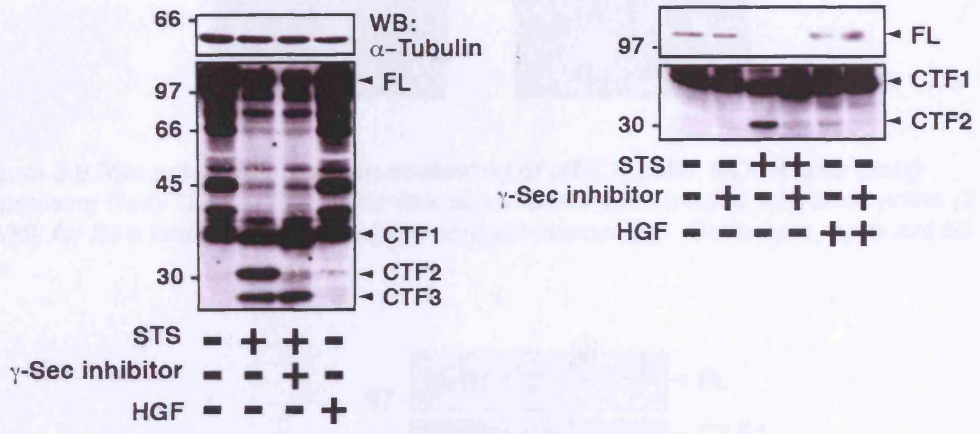


Figure 3.5 ***HGF induces γ-secretase–mediated cleavage of E-cadherin.*** *MDCK cells were treated with HGF (50 ng/ml) for 24 h or with staurosporine (1 μM) in the presence or absence of γ-secretase inhibitor X (12.5 nM) for 6 h. Cell lysates were examined by Western blotting with anti-E-cadherin and anti-α-tubulin antibodies. In right upper and lower panels, the film was exposed to the membrane after ECL for 10 seconds and 2 minutes, respectively. The positions of full length E-cadherin (FL), E-cad/CTF1, E-cad/CTF2, and E-cad/CTF3 are indicated with arrowheads. It should be noted that staurosporine treatment strongly reduces the amount of full length E-cadherin.*

3.3.3 Activation of Ras induces production of E-cad/CTF2 in MDCK cells

As described in section 1.4.1 (Introduction), activation of the small GTPase Ras also leads to transformation of epithelial cells resulting in the loss of cell-cell contacts (Schoenenberger et al., 1991). MDCK cells expressing a constitutively active form of Ras (RasV12) in a tetracycline-inducible manner were used to analyse the effect of Ras activation on E-cadherin cleavage. In the presence of tetracycline, these cells lost tight cell-cell contacts and started scattering (Figure 3.6). Under this condition, upregulation of E-cad/CTF2 was observed, although

to a lesser extent to that observed following HGF treatment (Figure 3.7). The amount of E-cad/CTF2 induced by Ras activation was not clearly affected by treatment with γ-secretase inhibitor X (Figure 3.7). Hence at this time the possibility cannot be excluded that there may be other proteases involved.

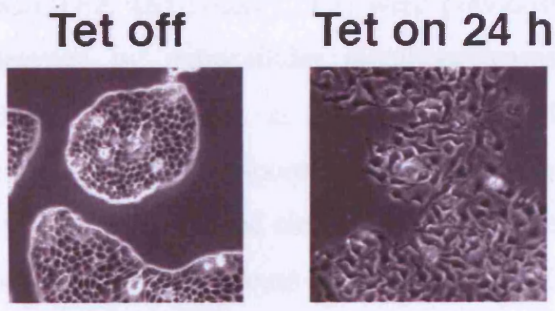


Figure 3.6 ***Ras activation leads to scattering of MDCK cells.*** *MDCK cells stably expressing RasV12 in a tetracycline-inducible manner were treated with tetracycline (2 μg/ml) for 24 h and examined by phase-contrast microscopy. Scale bars represent 50 μm.*

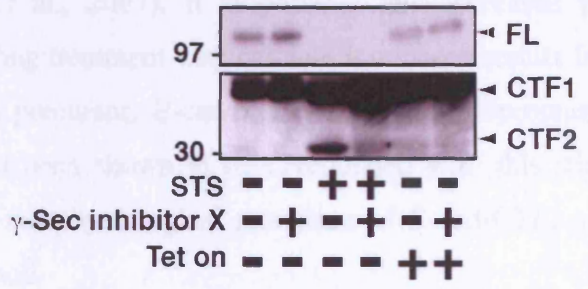


Figure 3.7 ***Ras activation increases production of E-cad/CTF2.*** *MDCK cells stably expressing RasV12 in a tetracycline-inducible manner were treated with tetracycline (2 μg/ml) for 24 h, or staurosporine (1 μM) in the presence or absence of γ-secretase inhibitor X (12.5 nM) for 6 h. Cell lysates were examined by Western blotting with anti-E-cadherin. In the upper and lower panels, the film was exposed to the membrane after ECL for 10 seconds and 2 minutes, respectively. The positions of full-length E-cadherin (FL), E-cad/CTF1 and E-cad/CTF2 are indicated with arrowheads. It should be noted that staurosporine treatment strongly reduces the amount of full length E-cadherin*

3.4 Discussion

This chapter describes an analysis of stimuli that lead to the production of the E-cadherin cleavage product, E-cad/CTF2. Three cleavage products of E-cadherin (E-cad/CTF1, E-cad/CTF2, and E-cad/CTF3) were previously characterised as resulting from cleavage by extracellular metalloproteases, γ-secretase and caspase-3 respectively (Marambaud et al., 2002). In this previous study Marambaud et al. reported that staurosporine treatment leads to the production of E-cad/CTF2 via a γ-secretase-mediated cleavage in A431 cells. In this chapter it is shown that this cleavage also occurs in MCF-7 and MDCK cells and is therefore not a cell-type specific event.

Other stimuli have been shown to induce production of E-cad/CTF2: treatment with a calcium ionophore or with the *Bacteroides fragilis* toxin (Marambaud et al., 2002; Wu et al., 2007). It is possible that increased production of E-cad/CTF2 following treatment with calcium ionophore results from the increased production of its precursor, E-cad/CTF1, since metalloprotease cleavage of E-cadherin has also been shown to be upregulated with this stimulus (Ito et al., 1999). However the physiological relevance of E-cad/CTF2 production is still not fully understood.

Although staurosporine induces apoptosis of cells, it also potently inhibits PKC and other kinases. Here, in order to analyse the effect of inducing apoptosis alone, UV irradiation was used. E-Cad/CTF2 was also produced following exposure to UV. This indicates that when cells undergo apoptosis, E-cadherin is cleaved to produce E-cad/CTF2. The disruption of E-cadherin based cell-cell contacts is involved in the onset of anoikis (Fouquet et al., 2004), and is an important process enabling apoptotic epithelial cells to round up and exit the epithelium (Steinhusen et al., 2001). Therefore it is possible that γ-secretase- and caspase-3-mediated cleavages of E-cadherin play a role in the down-regulation of E-cadherin from sites of cell-cell contacts during apoptosis.

Here, to study more physiological stimuli, it is shown that activation of the HGF-receptor leads to γ-secretase-dependent accumulation of E-cad/CTF2 in MDCK cells. As described in section 1.4.1 (Introduction), HGF, also known as scatter factor, is a paracrine morphogenic factor which regulates cellular growth, motility and invasiveness (Stoker and Gherardi, 1991; Weidner et al., 1990). It is known that upon stimulation of MDCK cells with HGF, endocytosis of E-cadherin is induced, leading to the disruption of cell-cell contacts (Fujita et al., 2002; Kamei et al., 1999; Weidner et al., 1990). Enhanced production of E-cad/CTF2 was also observed by activation of Ras, which also induces disruption of E-cadherin-based cell-cell contacts (Schoenenberger et al., 1991). Collectively, these data suggest that when E-cadherin is released from cell-cell contacts, it may become more accessible to proteases. The amount of E-cad/CTF2 induced by activation of the HGF-receptor or Ras is much smaller than that of full-length E-cadherin. This observation is quite comparable to γ-secretase-induced cleavage of Notch. Upon binding with its ligand, Notch is cleaved by γ-secretase to release its intracellular domain, however the amount is too small to be detected in the nucleus by immunostaining (Struhl and Adachi, 1998; Struhl and Greenwald, 1999). Thus, the main evidence of nuclear localisation of Notch has been presented with a reporter assay using a Notch construct C-terminally tagged with GAL4-VP16 (Struhl and Adachi, 1998). To demonstrate the physiological significance of E-cad/CTF2 *in vivo*, a similar reporter system needs to be applied in the future.

To investigate a potential nuclear signalling role of E-cad/CTF2, as has been described for intracellular domain fragments released by γ-secretase-cleavage of other transmembrane proteins, the fate of this fragment was studied. Chapter 4 describes the analysis of the fate of this fragment using E-cad/CTF2 expression studies.

CHAPTER 4

4 p120 ENHANCES THE NUCLEAR LOCALISATION OF E-CAD/CTF2

4.1 Introduction

The fate of E-cad/CTF2, following its release into the cytosol after cleavage of full length E-cadherin, was examined in this chapter. In particular it was investigated whether E-cad/CTF2 can localise in the nucleus, as has been observed for the γ-secretase-cleavage products of N-cadherin and γ-protocadherins (Haas et al., 2005; Hambsch et al., 2005; Shoval et al., 2007; Uemura et al., 2006a). Despite extensive efforts (for more details see Section 4.5.1), it has not been possible to isolate pure nuclear fractions from the epithelial cells A431, MCF-7 or MDCK, thus the nuclear localisation of endogenous E-cad/CTF2 could not be biochemically determined. Furthermore, the available antibodies against the cytoplasmic domain of E-cadherin are not suitable for immunofluorescent staining. In this chapter, expression studies using E-cadherin constructs were employed. Using three different approaches, employing both cell biology and biochemical techniques, a crucial and specific role for p120 in the nuclear localisation of E-cad/CTF2 was demonstrated.

4.2 Constructs used for expression studies

The constructs used for the expression studies in this chapter are outlined in figure 4.1. Firstly, a HA-tagged form of E-cad/CTF2 (Figure 4.1A) was used to examine the fate of this fragment when expressed in several cell lines by immunofluoresence microscopy. Secondly, MDCK cells expressing C-terminally myc-tagged full length E-cadherin (Figure 4.1B) were used. Following the induction of E-cadherin cleavage, the use of this constuct allows the fate of cleaved intracellular E-cadherin to be examined by immunofluoresence microscopy. Finally, constructs were produced of both of the fragments released into the cytosol by E-cadherin cleavage, E-cad/CTF2 (Figure 4.1 C) and E-cad/CTF3 (Figure 4.1D), for use in biochemical fractionation studies. E-cad/CTF2 and E-cad/CTF3 represent amino acids 734-884 and 753-884 of E-

cadherin, respectively. These constructs were produced according to the published cleavage sites of γ-secretase and caspase-3, respectively (Marambaud et al., 2002). A mutant form of E-cad/CTF2 was also constructed: E-cad/CTF2(AAA) (Figure 4.1E). This contains a triple alanine mutation within the CH2 domain which blocks the binding of E-cadherin with p120 (Thoreson et al., 2000).

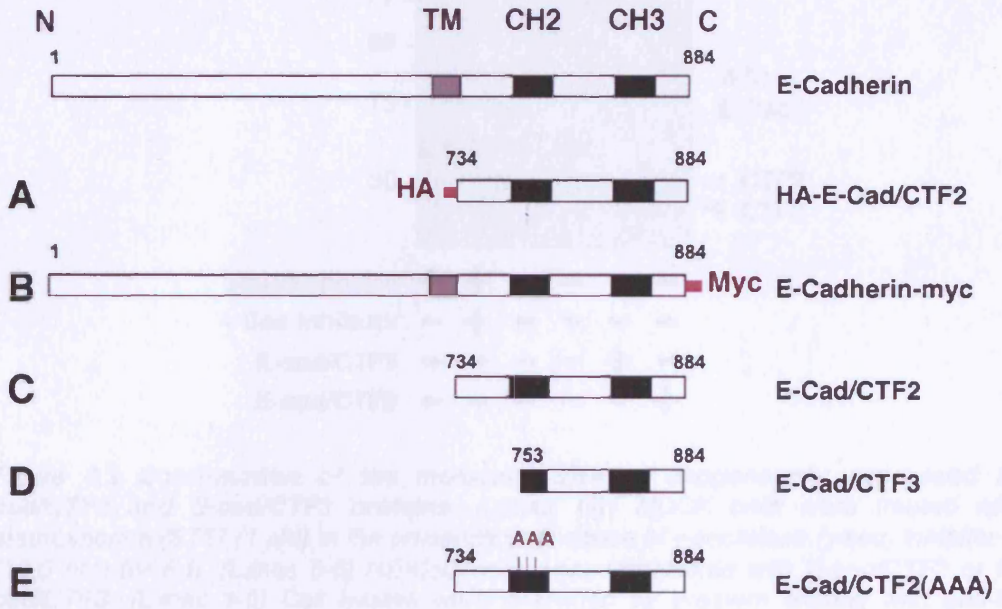


Figure 4.1 ***Schematics of the constructs used in expression studies in this chapter.*** *(A) HA-E-Cad/CTF2 construct. Amino acids 734-884 of E-cadherin with an N-terminal HA tag. (B) E-Cadherin-myc construct. Full length of E-cadherin with a C-terminal myc tag. (C) E-Cad/CTF2 construct. Amino acids 734-884 of E-cadherin. (D) E-Cad/CTF3 construct. Amino acids 753-884 of E-cadherin. (E) E-Cad/CTF2(AAA) construct. Amino acids 734-884 of E-cadherin with a triple alanine mutation within the CH2 domain. CH; cadherin homology domains. TM; transmembrane region.*

To confirm that the E-cad/CTF2 and E-cad/CTF3 constructs (shown in Figure 4.1C and 4.1D) were of the same molecular size as the endogenously produced E-cadherin cleavage products, cell lysates from HEK293 cells transfected with E-cad/CTF2 and E-cad/CTF3 were subjected to SDS-PAGE together with cell lysates from MDCK cells that had been treated with staurosporine with and without γ-secretase inhibitor X (Figure 4.2). As described in chapter 3, treatment of MDCK cells with staurosporine induced production of E-cad/CTF2 and E-cad/CTF3 (Figure 4.2), and γ-secretase inhibitor X blocked the production of E-cad/CTF2 (Figure 4.2). Comparison of the Western blot bands showed that exogenous E-cad/CTF2 and E-cad/CTF3 expressed in HEK293 cells were of the

same molecular size as those produced by cleavage of endogenous E-cadherin in MDCK cells (Figure 4.2).

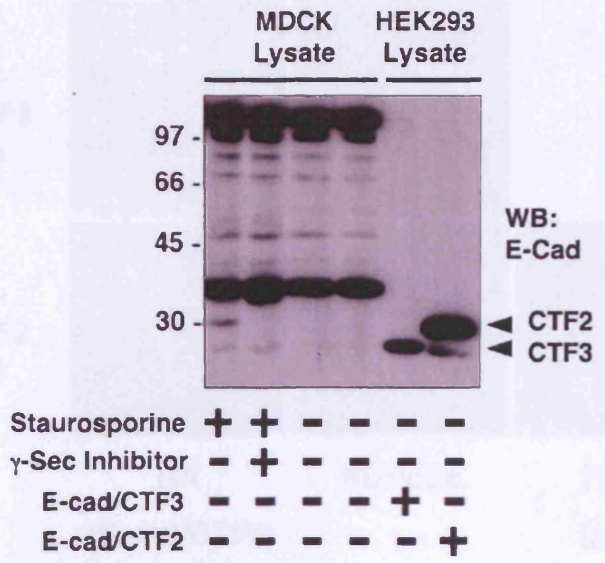
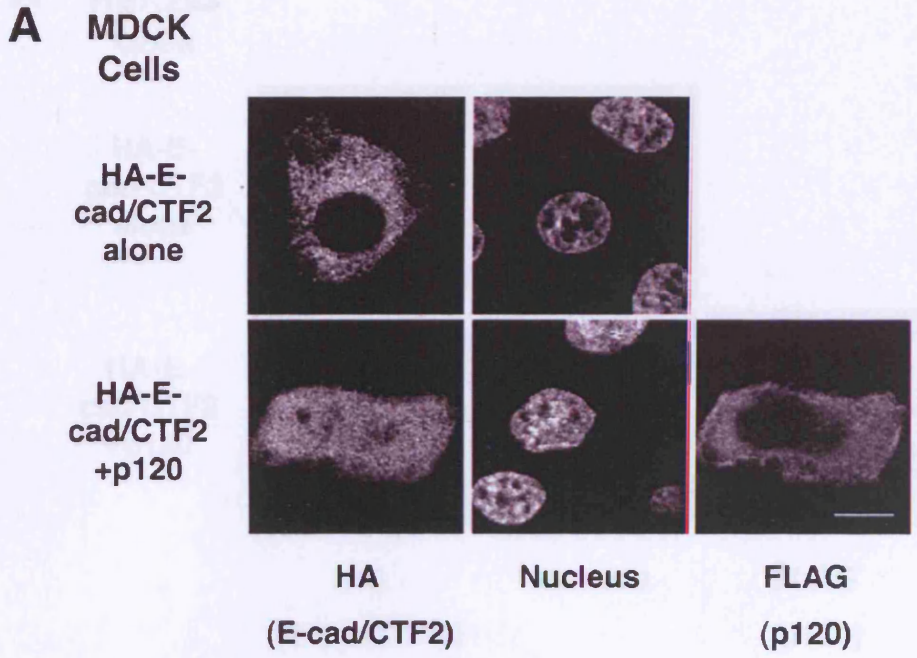


Figure 4.2 ***Confirmation of the molecular size of exogenously expressed E-cad/CTF2 and E-cad/CTF3 proteins.*** *(Lanes 1-2) MDCK cells were treated with staurosporine (STS) (1 μM) in the presence or absence of γ-secretase (γ-sec) inhibitor X (12.5 nM) for 6 h. (Lanes 5-6) HEK293 cells were transfected with E-cad/CTF2 or E-cad/CTF3. (Lanes 1-6) Cell lysates were examined by Western blotting with anti-E-cadherin antibody. The positions of E-cad/CTF2, and E-cad/CTF3 are indicated with arrowheads.*

4.3 p120 enhances nuclear localisation of E-cad/CTF2

The HA-tagged E-cad/CTF2 construct described in Figure 4.1B was expressed in epithelial cells and its subcellular localisation was examined. When HA-E-cad/CTF2 alone was expressed in MDCK cells, it mainly localised in the cytoplasm (Figure 4.3A). Interestingly, coexpression of p120 enhanced the nuclear localisation of HA-E-cad/CTF2 (Figure 4.3A). To quantify this effect of p120, the ratio of pixel intensity was measured within defined regions (diameter 1.97 μm) of the nucleus and cytoplasm. The quantification results showed that coexpression of p120 significantly increased the amount of E-cad/CTF2 in the nucleus ($p < 0.002$) (Figure 4.3B). A similar effect of p120 on the nuclear localisation of E-cad/CTF2 was observed in human embryonic kidney (HEK) 293 cells (Figure 4.4) and also MCF-7 cells (Figure 4.5)



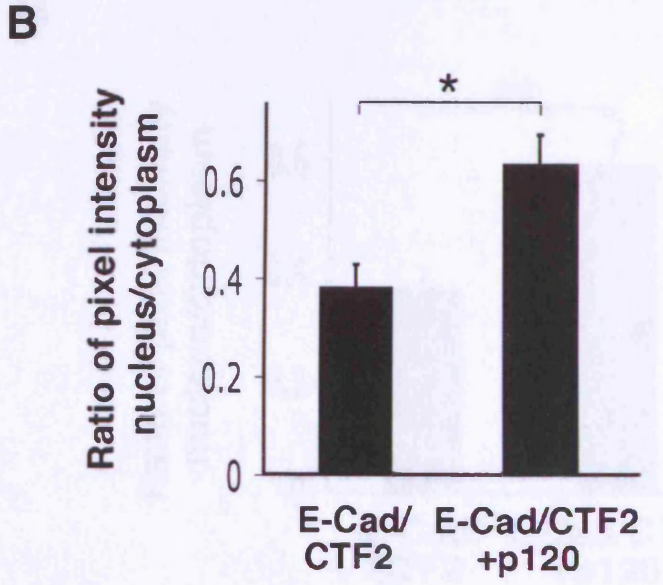


Figure 4.3 ***p120 enhances the nuclear localisation of E-cad/CTF2 in MDCK cells.*** *(A) MDCK cells were transfected with HA-E-cad/CTF2 and FLAG-p120. Immunostaining was performed with anti-HA and anti-FLAG antibodies. Nuclei were stained with Hoechst dye. Images were acquired using confocal microscopy. Scale bars represent 10 μm. (B) Quantification of p120-induced enhanced nuclear localisation of HA-E-cad/CTF2 in MDCK cells. Immunofluorescence intensity with anti-HA antibody was measured within a defined region (diameter 1.97 μm) in the nucleus and cytoplasm. The ratio of nucleus/cytoplasm staining intensity was analysed in 20 cells. Error bars represent SEM. *, $p < 0.002$.*

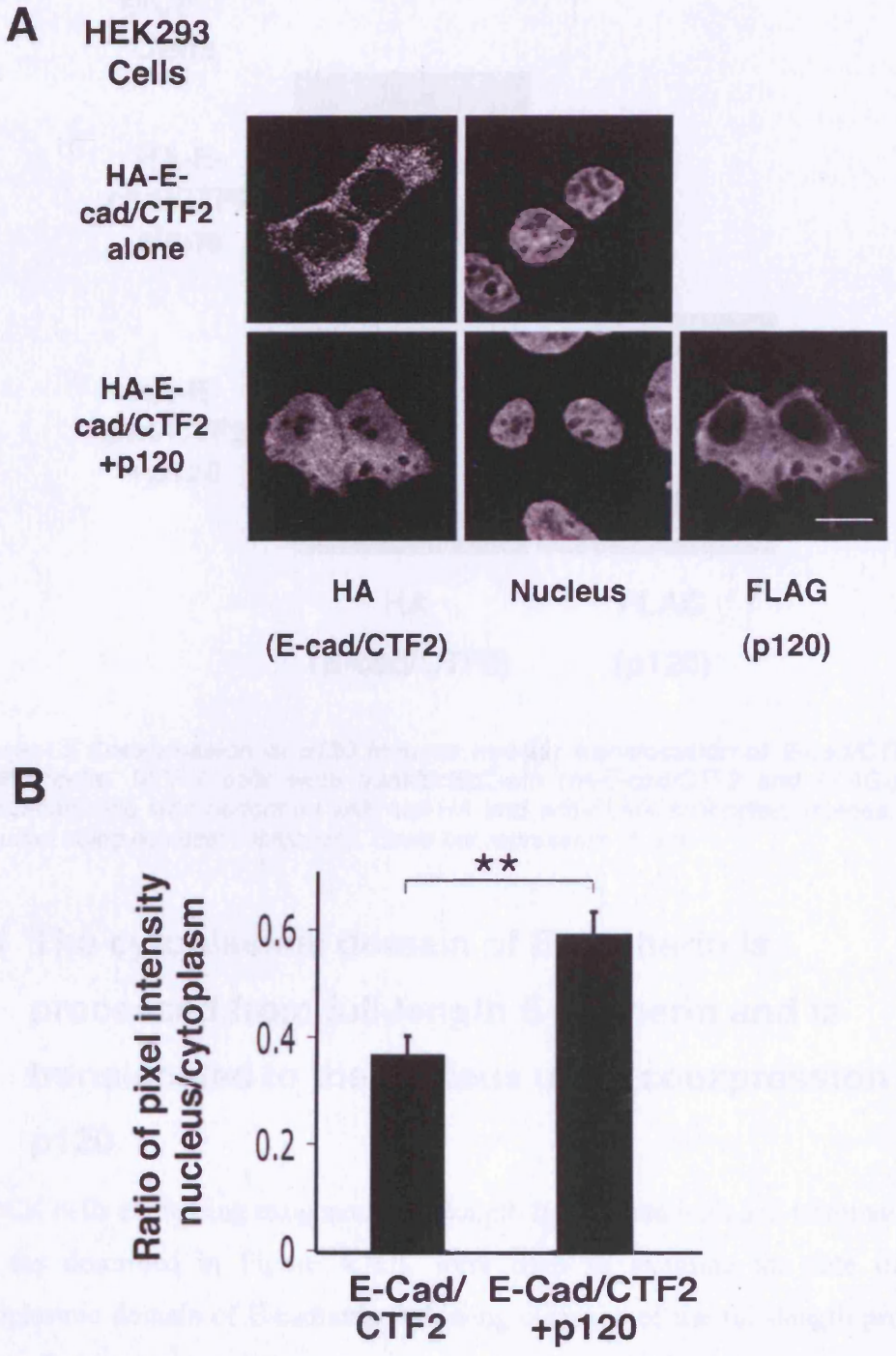


Figure 4.4 ***p120 enhances the nuclear localisation of E-cad/CTF2 in HEK293 cells.*** *(A) HEK293 cells were transfected with HA-E-cad/CTF2 and FLAG-p120. Immunostaining was performed with anti-HA and anti-FLAG antibodies. Nuclei were stained with Hoechst dye. Images were acquired using confocal microscopy. Scale bars represent 10 μm. (B) Quantification of p120-induced enhanced nuclear localisation of HA-E-cad/CTF2 in HEK293 cells. Immunofluorescence intensity with anti-HA antibody was measured within a defined region (diameter 1.97 μm) in the nucleus and cytoplasm. The ratio of nucleus/cytoplasm staining intensity was analysed in 20 cells. Error bars represent SEM. **, $p < 0.00007$.*

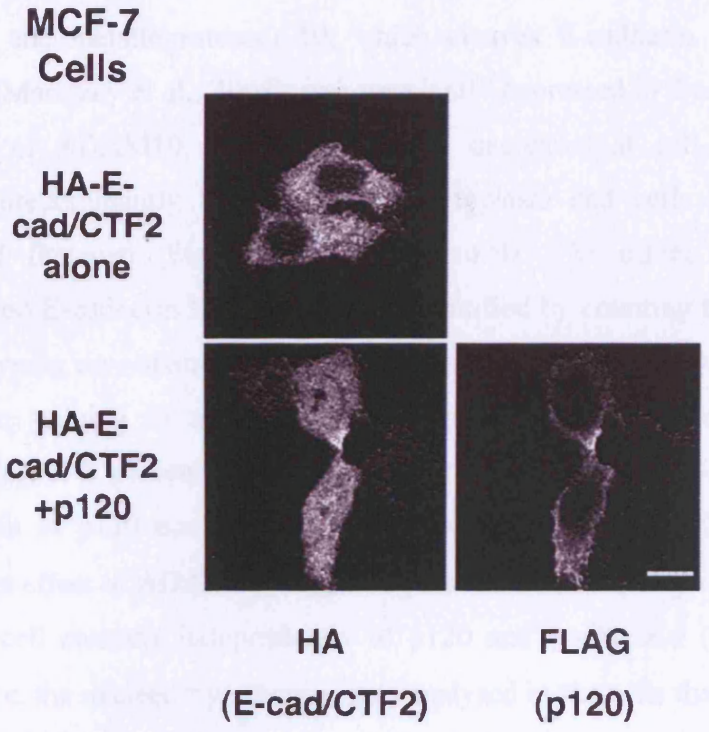


Figure 4.5 ***Coexpression of p120 induces nuclear translocation of E-cad/CTF2 in MCF-7 cells.*** *MCF-7 cells were transfected with HA-E-cad/CTF2 and FLAG-p120. Immunostaining was performed with anti-HA and anti-FLAG antibodies. Images were acquired using confocal microscopy. Scale bar represents 15 μm.*

4.4 The cytoplasmic domain of E-cadherin is processed from full-length E-cadherin and is translocated to the nucleus upon coexpression of p120

MDCK cells expressing exogenous full-length E-cadherin with a C-terminal myc tag (as described in Figure 4.1C), were used to examine the fate of the cytoplasmic domain of E-cadherin following cleavage of the full-length protein. When E-cadherin-myc alone was expressed, myc immunostaining was mainly observed at cell-cell contacts, which was not affected by coexpression of p120 (Figure 4.6A, first and second panels). It was next investigated whether the cleaved C-terminus of E-cadherin localises in the nucleus in this system. Staurosporine treatment induced nuclear fragmentation and therefore was not a suitable treatment to analyse nuclear localisation of cleaved E-cadherin. The amount of E-cad/CTF2 induced by HGF treatment was too small to be detected

with immunostaining. Thus as an alternative, a metalloprotease ADAM (a disintegrin and metalloprotease) 10, which cleaves E-cadherin producing E-cad/CTF1 (Maretzky et al., 2005), was transiently expressed in these cells. Upon expression of ADAM10, myc staining was decreased at cell-cell contacts, becoming predominantly localised in the cytoplasm and cells became more spread and flattened (Figure 4.6A, third panel). The effect of ADAM10 expression on E-cadherin localisation was quantified by counting the percentage of cells showing myc staining in the cytoplasm. Expression of ADAM10 lead to cytoplasmic staining in approximately 60 percent of cells, compared with approximately 10 percent in mock-transfected cells (Figure 4.6B). Neither coexpression of p120 nor treatment with γ-secretase inhibitor X significantly affected this effect of ADAM10, suggesting that ADAM10 disrupted E-cadherin-based cell-cell contacts independently of p120 and γ-secretase (Figure 4.6B). Furthermore, the nuclear myc staining was analysed in the cells that lost cell-cell contact staining upon expression of ADAM10. Notably, coexpression of p120 with ADAM10 substantially increased nuclear myc staining (Figure 4.6A, fourth panel, and Figure 4.6C). The nuclear myc staining induced by coexpression of ADAM10 and p120 was blocked by treatment with γ-secretase inhibitor X (Figure 4.6A, fifth panel, and Figure 4.6C), suggesting that the nuclear localisation of the C-terminus of E-cadherin is dependent on γ-secretase-mediated processing of E-cad/CTF1. Collectively, data in sections 4.3 and 4.4 indicate that the γ-secretase cleavage product of E-cadherin, E-cad/CTF2, can localise in the nucleus of epithelial cells and that this localisation is enhanced by p120.

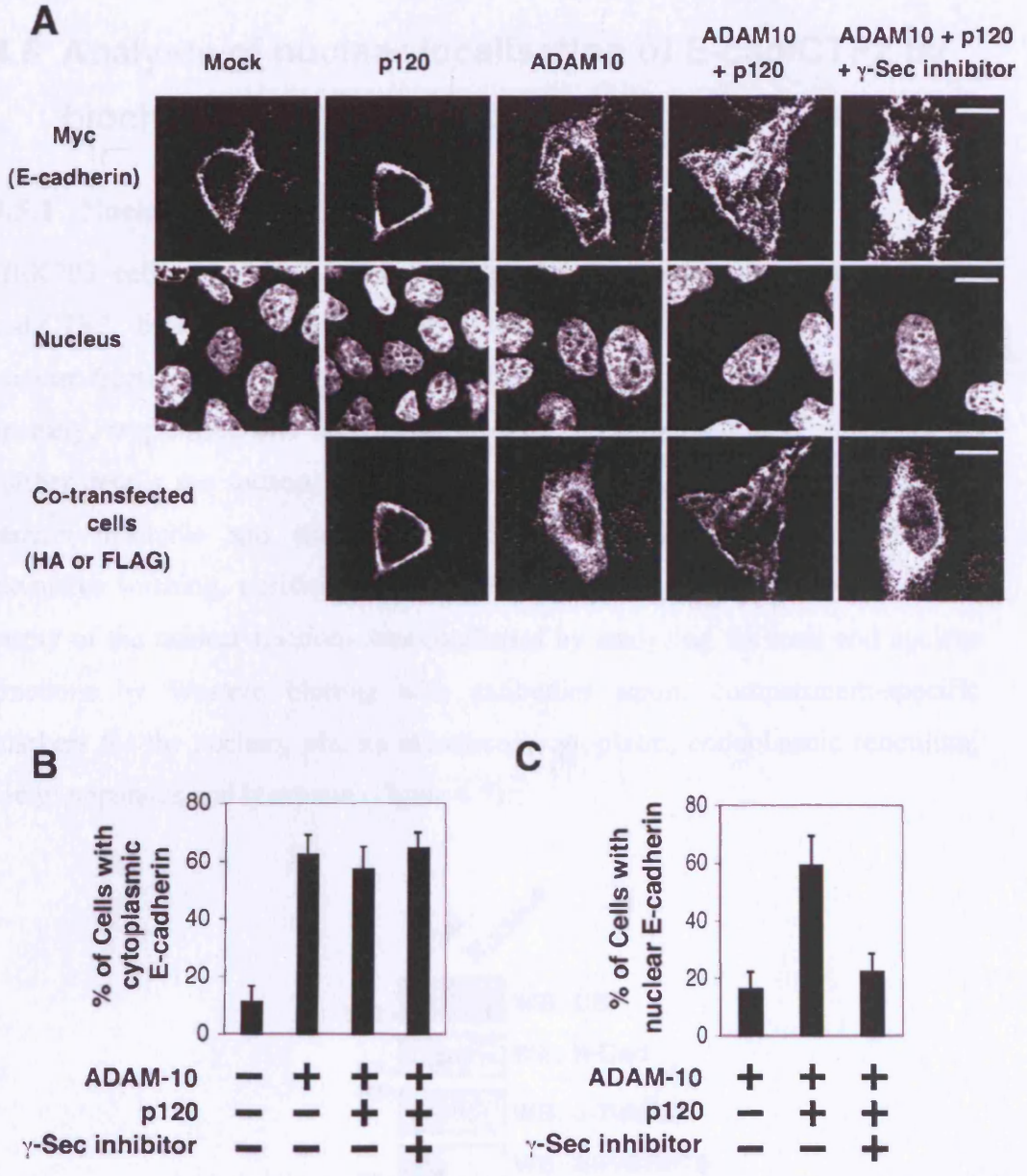


Figure 4.6 ***Nuclear localisation of the cleaved C-terminus of E-cadherin.*** *(A) p120 induces nuclear localisation of the C-terminus of E-cadherin in ADAM10-transfected cells. MDCK cells stably expressing C-terminally myc-tagged E-cadherin were transiently transfected with FLAG-ADAM10 and/or HA-p120. γ-Secretase inhibitor X (12.5 nM) was added for 6 h before fixation where indicated. Immunostaining was performed with anti-myc, anti-HA, or anti-FLAG antibodies. Nuclei were stained with Hoechst dye. Images were acquired using confocal microscopy. Scale bars represent 15 μm. (B) Quantification of ADAM10-induced cytoplasmic localisation of E-cadherin. The percentage of cells showing cytoplasmic myc-staining was calculated from 20 transfected cells from two independent transfections. Error bars indicate SEM. (C) Quantification of nuclear localisation of E-cadherin. The percentage of cells showing loss of myc staining at cell-cell contacts that also showed nuclear myc staining was calculated from 20 transfected cells from two independent transfections. Error bars indicate SEM.*

4.5 Analysis of nuclear localisation of E-cad/CTF2 by biochemical nuclear fractionation in HEK293 cells.

4.5.1 Nuclear fractionation in HEK293 cells

HEK293 cells were used to further analyse the nuclear localisation of E-cad/CTF2, because highly purified nuclei can be obtained by biochemical nuclear fractionation using these cells. In this technique the cells were plated sparsely, trypsinised and then lysed in a buffer containing 20% glycerol (for further details see section 2.3.8, Chapter 2). Under these conditions the nuclei remain insoluble and can be pelleted following centrifugation, and after extensive washing, purified nuclei can be observed by light microscopy. The purity of the nuclear fractions was confirmed by analyzing the total and nuclear fractions by Western blotting with antibodies agains compartment-specific markers for the nucleus, plasma membrane, cytoplasm, endoplasmic reticulum, Golgi apparatus and lysosome (Figure 4.7).

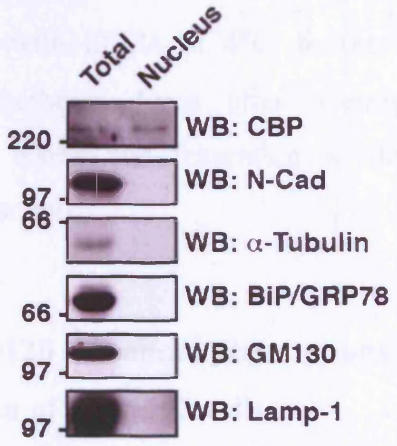


Figure 4.7 ***Characterization of nuclear fractions isolated from HEK293 cells.*** *Total and nuclear lysates from HEK293 cells were examined by Western blotting with antibodies against different subcellular compartments: anti-CREB binding protein (CBP) (nucleus); anti-N-cadherin (plasma membrane); anti-α-tubulin (cytoplasm); anti-BiP/GRP78 (endoplasmic reticulum); anti-GM130 (Golgi); anti-Lamp-1 (lysosome).*

This technique for nuclear fractionation was not suitable for the epithelial cell lines A431, MCF-7 or MDCK. This is because cell aggregates were not well

separated with trituration in the lysis buffer, most likely due to the increased strength of cell-cell contacts in these cell lines. Thus the nuclear pellet was contaminated with cell aggregates and were observed in the resuspended pellet by light microscopy. Trypsinisation is often used to separate cell aggregates in nuclear fractionation techniques. However, trypsinisation leads to cleavage of E-cadherin (Perret et al., 2002), and so it would not be possible to analyse endogenous E-cadherin cleavage products after treating cells with trypsin.

Several other variations of this method were attempted to purify nuclear fractions from these three cell lines. Sonication was tested to break up the cell aggregates. However at the amplitudes required to disrupt the cell aggregates, nuclei were also disrupted and could no longer be pelleted by centrifugation. E-cadherin-based cell-cell contacts are calcium dependent and can be disrupted by treatment with ethylene glycol tetraacetic acid (EGTA). Addition of EGTA chelates the calcium ions and thus sequester the calcium ions away from E-cadherin (Reddy et al., 2005). Thus incubation with EGTA was also used in order to break up the cell aggregates. However since calcium regulates the cleavage of E-cadherin by metalloproteases and γ-secretase (Ito et al., 1999; Marambaud et al., 2002), it was necessary to incubate with EGTA at 4°C, to prevent the EGTA from affecting cleavage of E-cadherin. Even after overnight incubation, cell aggregates remained after lysing and trituration as described above, thus contaminating the nuclear fraction.

4.5.2 Coexpression of p120 enhances the amount of E-cad/CTF2 in the nuclear fraction of HEK293 cells

Since it has not been possible to determine whether the cleavage products of endogenous E-cadherin localise to the nucleus by examining nuclear fractions of A431, MCF-7 or MDCK cells, nuclear fractionation experiments were carried out in HEK293 cells. It was first confirmed that coexpression of p120 increased the amount of E-cad/CTF2 in the nuclear fraction, as is expected from the immunostaining data in HEK293 cells shown in Figure 4.4 (Figure 4.8, A and B). The expression of p120 did not affect the amount of nuclei in the nuclear fraction, indicated by levels of CBP in the nuclear fraction (Figure 4.8B).

Interestingly, it was also observed that p120 expression frequently induced a series of higher molecular weight modifications of E-cad/CTF2 (Figure 4.8A, 'upsmear' of modifications indicated by double-headed arrow). Studies of this modification of E-cad/CTF2 are presented in chapter 5. It should be noted that the extent of modifications varied between experiments (compare Figure 4.8A with 4.8B). See section 5.6 for further discussion of the modifications of E-cad/CTF2 and the variability in the amount of modification between experiments.

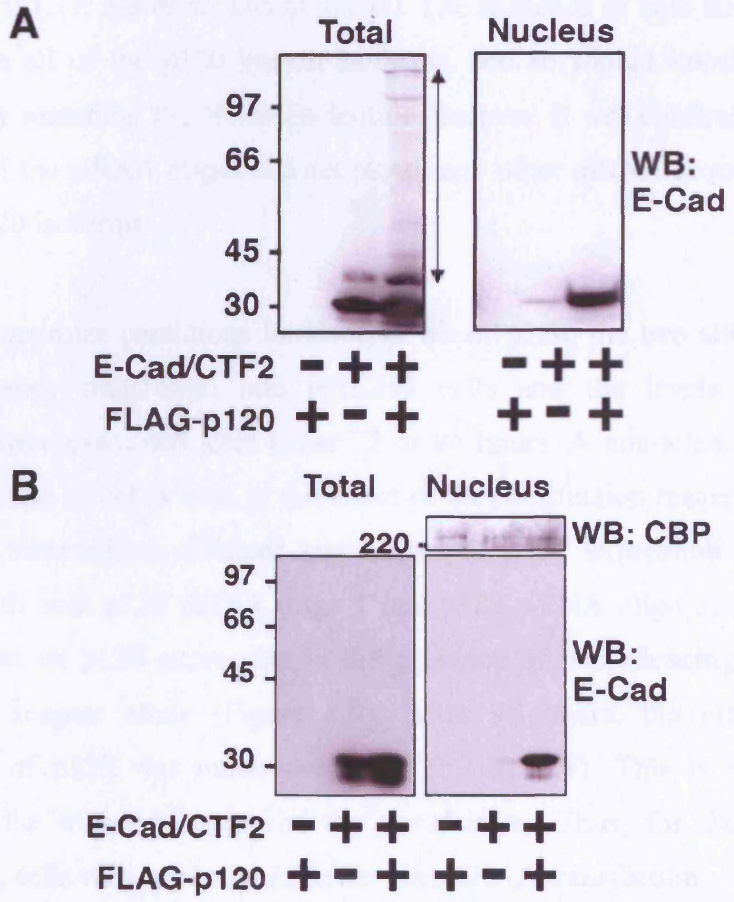


Figure 4.8 ***Coexpression of p120 increases the amount of E-cad/CTF2 in the nuclear fraction without affecting the amount of nuclei in this fraction.*** *(A and B) HEK293 cells were transfected with E-cad/CTF2 and FLAG-p120. Total and nuclear lysates were examined by Western blotting with anti-E-cadherin antibody. (A) By kind permission of Dr. Yasuyuki Fujita. Double headed arrow indicates 'upsmear' of modifications induced by p120. (B) Nuclear lysates were also examined by Western blotting with anti-CBP antibody.*

4.5.3 Knockown of p120 using small interfering RNA oligonucleotides

To investigate whether p120 is required for the nuclear localisation of E-cad/CTF2 in HEK293 cells, p120 protein was knocked down using small interfering RNA (siRNA) oligonucleotides (oligos). Knockdown of gene expression levels with this technique is achieved by the activation of the RNA-induced silencing complex (RISC) which destroys mRNAs that contain the identical sequence found in the siRNA oligo (Sen and Blau, 2006). Using the Eurogentec web-based ordering system, two siRNA oligos were designed (Table 2.6, Section 2.1.11, Materials and Methods). The sequence of both siRNA oligos are found in all of the p120 human isoforms, and so should knock down all isoforms. By searching the NCBI nucleotide database, it was confirmed that the sequences of the siRNA oligos did not match any other mRNA sequences apart from the p120 isoforms.

In order to optimize conditions for knockdown of p120, the two siRNA oligos were transiently transfected into HEK293 cells and the levels of protein expression were examined after either 72 or 96 hours. A non-silencing siRNA control was also tested as well as the effect of the transfection reagent alone. 72 hours after transfection, efficient knockdown of p120 expression levels was achieved with both p120 siRNA oligo 1 and p120 siRNA oligo 2, while there was no effect on p120 expression in the presence of non-silencing siRNA or transfection reagent alone (Figure 4.9). After 96 hours, the effect of the knockdown of p120 was much decreased (Figure 4.9). This is most likely because of the transient nature of the transfection. Thus, for the following experiments, cells were analysed 72 hours after siRNA transfection.

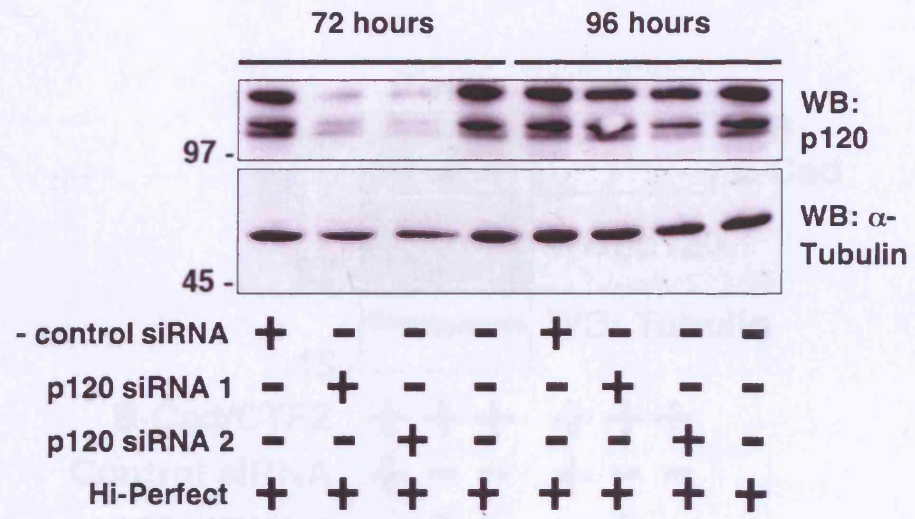


Figure 4.9 ***Optimisation of p120 knockdown by transient transfection of siRNA oligos.*** *HEK293 cells were transfected with negative (-) control siRNA, p120 siRNA 1, p120 siRNA 2 or treated with Hi-Perfect transfection reagent alone. 72 or 96 hours after transfection, total lysates were examined by Western blotting with anti-p120 and anti-α-tubulin antibodies.*

4.5.4 p120 is required for nuclear localisation of E-cad/CTF2 in HEK293 cells

The effect of knocking down the expression of endogenous p120 on the nuclear localisation of E-cad/CTF2 was examined. Expression of siRNA oligos efficiently knocked down the expression of endogenous p120 and strongly reduced the amount of E-cad/CTF2 present in the nuclear fraction (Figure 4.10). This reduction of E-cad/CTF2 in the nuclear fraction was observed to a greater extent with siRNA oligo 2 where greater knockdown of p120 was observed (Figure 4.10). These data indicate that p120 is required for the nuclear localisation of E-cad/CTF2 in HEK293 cells.

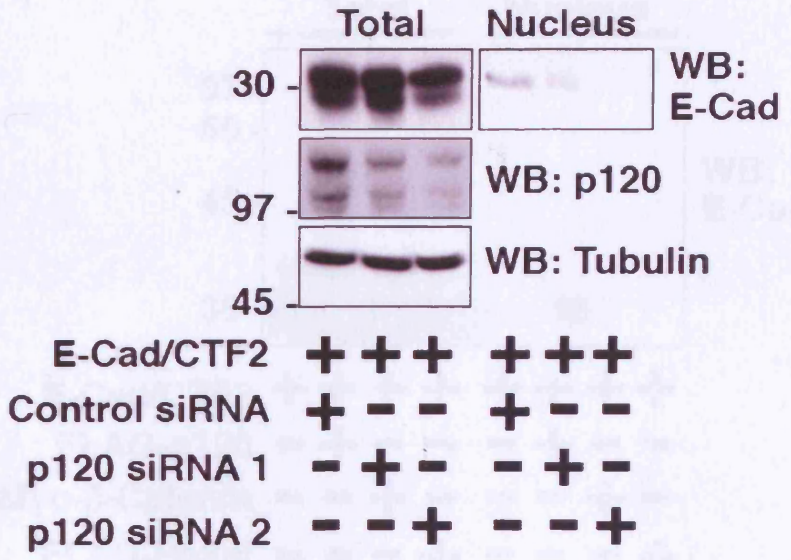


Figure 4.10 ***Knockdown of p120 decreases the nuclear localisation of E-cad/CTF2.*** *HEK293 cells were transfected with control siRNA, p120 siRNA oligo 1, or p120 siRNA oligo 2 together with E-cad/CTF2, and total and nuclear lysates were examined by Western blotting with anti-E-cadherin, anti-p120 and anti-α-tubulin antibodies.*

4.5.5 The nuclear localisation of E-cad/CTF2 is enhanced specifically by p120

Next the specificity of the effect of p120 on nuclear localisation and modification of E-cad/CTF2 was investigated. As described in section 1.2.2 (Introduction), the intracellular domain of E-cadherin binds to other proteins. Expression of two other E-cadherin binding proteins, β-catenin and Hakai, did not enhance either modification or nuclear localisation of E-cad/CTF2 (Figure 4.11). This suggests that these effects are specific to p120.

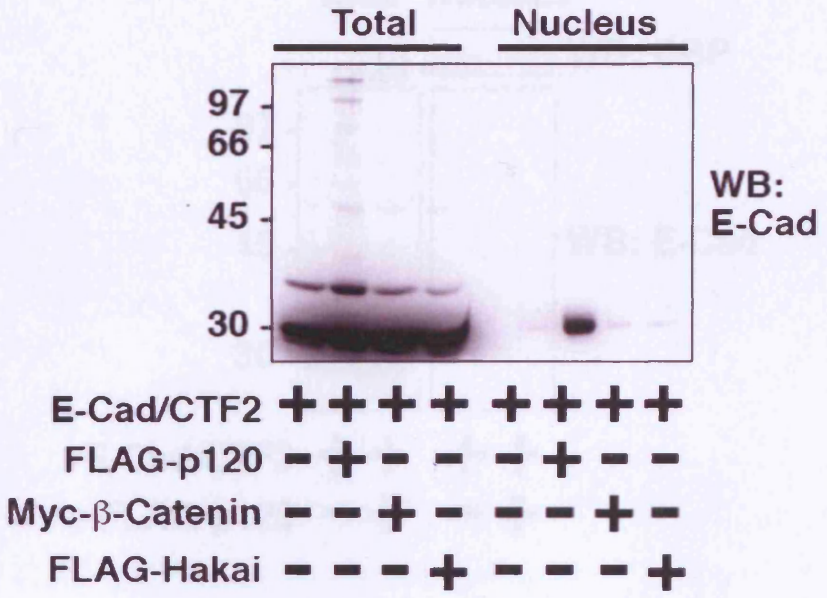


Figure 4.11 ***E-Cad/CTF2 nuclear localisation is increased by p120, but not by other E-cadherin binding proteins.*** *HEK293 cells were transfected with E-cad/CTF2 together with FLAG-p120, Myc-β-catenin or FLAG-Hakai, and total and nuclear lysates were examined by Western blotting with anti-E-cadherin. By kind permission of Dr. Yasuyuki Fujita.*

4.5.6 p120 does not induce nuclear localisation of E-cad/CTF3.

To further examine the specificity of the effect of p120 on inducing nuclear localisation and modification of E-cad/CTF2, the effect of p120 on another E-cadherin fragment, E-cad/CTF3, that is released into the cytosol following cleavage by caspase-3, was investigated. In this experiment the construct of E-cad/CTF3 described in Figure 4.1D was used. When E-cad/CTF3 alone was expressed, it was not detected in the nuclear fraction (Figure 4.12). The expression of p120 enhanced neither nuclear localisation nor modification of E-cad/CTF3 (Figure 4.12). This suggests that effect of p120 on the enhancement of nuclear localisation and modification is specific to E-cad/CTF2.

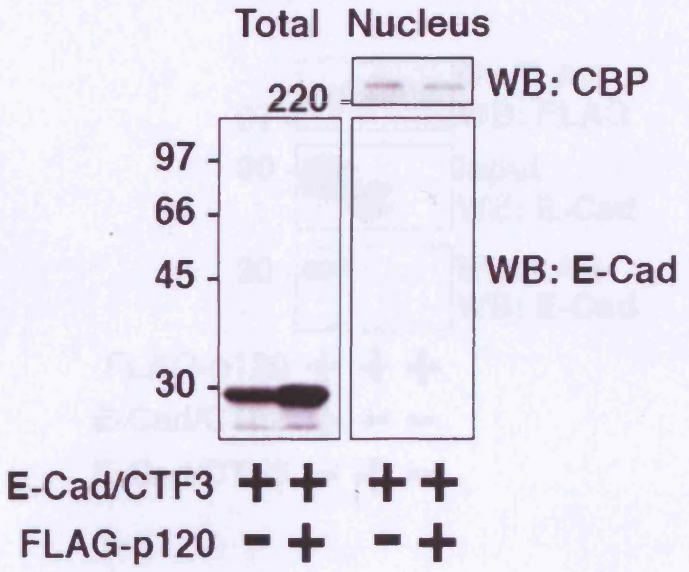


Figure 4.12 ***p120 does not induce nuclear localisation of E-cad/CTF3.*** *HEK293 cells were transfected with E-cad/CTF3 with and without FLAG-p120, and total and nuclear lysates were examined by Western blotting with anti-E-cadherin. Nuclear fractions were also examined by Western blotting with anti-CREB binding protein (CBP) antibody.*

4.5.7 E-Cad/CTF3 does not bind to p120

Since caspase-3 cleavage occurs within the CH2 domain that includes the p120-binding site, this cleavage may impair binding of E-cad/CTF3 to p120. To examine this possibility, the interaction between p120 and E-cad/CTF2 or E-cad/CTF3 was analysed by coimmunoprecipitation. Indeed it was found that E-cad/CTF3 was not coimmunoprecipitated with p120 while E-cad/CTF2 was (Figure 4.13). Together with the data in Figure 4.12, this suggests that p120 binding is required for the nuclear localisation of the E-cadherin intracellular domain.

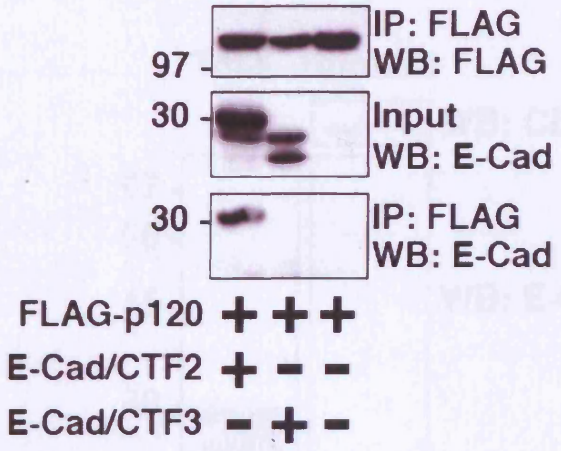


Figure 4.13 ***E-Cad/CTF3 does not bind to p120****. FLAG-p120 was transfected with E-cad/CTF2 or E-cad/CTF3 in HEK293 cells. Immunoprecipitation was performed using anti-FLAG antibody, followed by Western blotting with anti-E-cadherin and anti-FLAG antibodies.*

4.5.8 p120-binding is required for the nuclear localisation of E-cad/CTF2

To further investigate the requirement of p120 binding for its enhancement of E-cad/CTF2 nuclear localisation, a mutant form of E-cad/CTF2 which cannot bind to p120 (E-cad/CTF2(AAA)) was used (described in Figure 4.1E). This is a triple alanine mutation within the CH2 domain that prevents binding of E-cadherin to p120 (Thoreson et al., 2000). E-Cad/CTF2(AAA) was not present in the nuclear fraction and expression of p120 did not induce nuclear localisation or modification of E-cad/CTF2(AAA) (Figure 4.14). These data suggest that the direct interaction with E-cad/CTF2 is required for p120 to enhance nuclear localisation and modification of E-cad/CTF2.

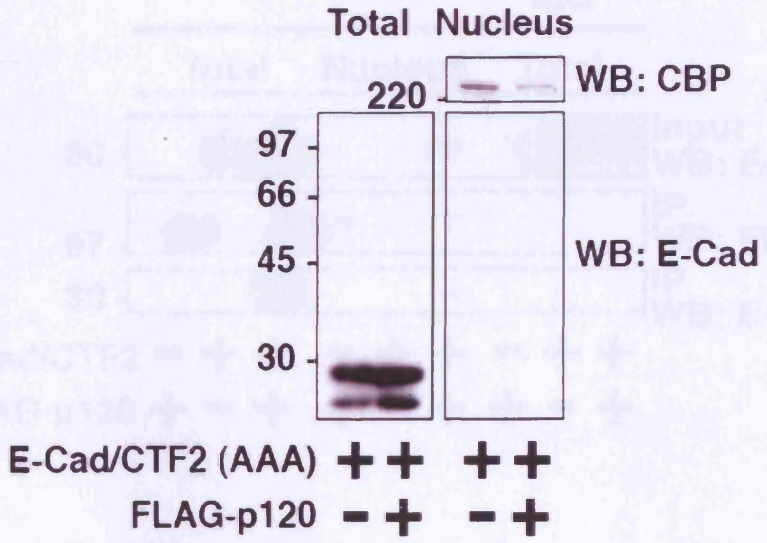


Figure 4.14 ***p120 binding is required for nuclear translocation of E-cad/CTF2.*** *HEK293 cells were transfected with E-cad/CTF2(AAA) with or without FLAG-p120 and total and nuclear lysates were examined by Western blotting with anti-E-cadherin antibody. Nuclear fractions were also examined by Western blotting with anti-CREB binding protein (CBP) antibody.*

4.5.9 E-Cad/CTF2 binds to p120 in the nucleus

Figure 4.13 shows by coimmunoprecipitation that E-cad/CTF2 and p120 bind to each other when expressed in HEK293 cells. In order to determine whether E-cad/CTF2 and p120 also interact with each other in the nucleus, coimmunoprecipiation was perfomed using nuclear fractions from HEK293 cells transiently transfected with E-cad/CTF2 and p120 (Figure 4.15). Small amounts of exogenously expressed p120 were detected in the nucleus, and E-cad/CTF2 was coimmunoprecipitated with p120 from the nuclear fraction (Figure 4.15). This suggests that p120 and E-cad/CTF2 interact with each other in the nucleus.

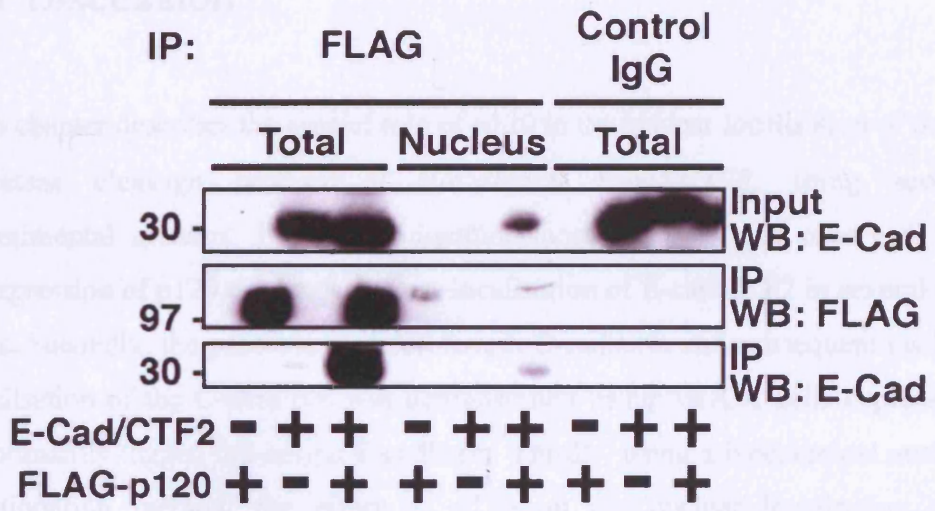


Figure 4.15 ***E-Cad/CTF2 and p120 interact in the nucleus.*** *Lysates or nuclear fractions of HEK293 cells expressing E-cad/CTF2 and FLAG-p120 were immunoprecipitated with anti-FLAG antibody or control IgG, followed by Western blotting with anti-E-cadherin and anti-FLAG antibodies.*

4.6 Discussion

This chapter describes the crucial role of p120 in the nuclear localisation of the γ-secretase cleavage product of E-cadherin, E-cad/CTF2, using several experimental systems. Firstly, by immunofluorescence it was observed that coexpression of p120 enhances nuclear localisation of E-cad/CTF2 in several cell lines. Secondly, the processing of full-length E-cadherin and subsequent nuclear localisation of the C-terminus was demonstrated using MDCK cells expressing C-terminally tagged full-length E-cadherin. Thirdly, using a biochemical nuclear fractionation method, the effect of p120 on the nuclear localisation was confirmed and it was shown that this effect is specific for p120 as other cadherin-binding proteins do not induce nuclear translocation of E-cad/CTF2. The requirement of p120 in E-cad/CTF2 nuclear localisation was also demonstrated by using siRNA oligos that knock down endogenous p120. The direct interaction between p120 and E-cad/CTF2 is also required because E-cad/CTF2(AAA) is not detected in the nuclear fraction.

The requirement for the direct binding of p120 for the nuclear localisation of E-cad/CTF2 suggests that p120 must be free from its role at the adherens junction in order to play this role in the nuclear translocation of E-cad/CTF2. p120 is sequestered in multiple cellular locations including adherens junctions and microtubules (Chen et al., 2003; Franz and Ridley, 2004; Reynolds et al., 1994; Yanagisawa et al., 2004). It has been shown that under conditions where E-cadherin is down-regulated, p120 is released into the cytosol (Roczniak-Ferguson and Reynolds, 2003; van Hengel et al., 1999). Data in chapter 3 indicate that E-cad/CTF2 is produced by γ-secretase-mediated cleavage when cells are treated with HGF (Figure 3.5). HGF treatment of MDCK cells leads to the downregulation of E-cadherin at cell-cell contacts both by endocytosis and by transcriptional downregulation (Fujita et al., 2002; Grotegut et al., 2006). This suggests that under conditions where E-cadherin at cell-cell contacts is being downregulated, E-cad/CTF2 is produced and p120 may be released into the cytosol. The requirement of direct binding to p120 suggests that regulation of

p120 subcellular localisation could therefore impact on the control of nuclear localisation of E-cad/CTF2.

The mechanism whereby p120 mediates nuclear translocation of E-cad/CTF2 is not clear. As shown in Figure 4.15, E-cad/CTF2 binds p120 in the nucleus, suggesting that the nuclear translocation of E-cad/CTF2 may be mediated directly by the stoichiometrical interaction with p120. E-cad/CTF2 does not contain any classical NLS sequences, but p120 is subject to nucleocytoplasmic shuttling (Roczniak-Ferguson and Reynolds, 2003; van Hengel et al., 1999), and so E-cad/CTF2 could potentially be carried in to the nucleus by p120. However, it is also possible that the effect of p120 is mediated catalytically, for example through post-translational modification of E-cad/CTF2.

As described in section 1.3.5 (Introduction), the control of p120 nuclear localisation is complex and not fully understood. p120 contains both NLS and NES sequences, although some data suggest that the Arm repeat regions are more important for the control of its nuclear localisation (Roczniak-Ferguson and Reynolds, 2003). As described in section 1.3.5 (Introduction), the Arm domain of Importin-α is its soley functional domain. β-catenin has been shown to bind directly to Importin-β, and it is possible that p120 could act similarly to promote its own nuclear localisation through its Arm domain. This idea raises the possibility that p120 could also promote the nuclear localisation of other proteins.

It should be noted that the nuclear localisation of p120 is not clearly affected by the presence of E-cad/CTF2, and p120 appears to be mainly localised in the cytoplasm even when E-cad/CTF2 is in the nucleus (Figures 4.3, 4.4, 4.5 and 4.15). This could be due to rapid nucleocytoplasmic shuttling of p120. p120 may remain localised in the nucleus only under certain conditions. For example, it was recently shown that expression of a p120-binding transcription factor Glis2, induces substantial nuclear accumulation of p120 (Hosking et al., 2007). Thus it is possible that E-cad/CTF2 is carried in to the nucleus via its association with p120, but then is not exported with p120. Indeed as only a small amount of p120

is observed in the nucleus under conditions where a large amount of E-cad/CTF2 is seen in the nucleus (Figure 4.15), some nuclear E-cad/CTF2 may dissociate from p120 under these conditions. This could be due to the binding of E-cad/CTF2 to other nuclear proteins that may allow its retention in the nucleus. In order to examine these possibilities, the effect of mutations in the NLS, NES, and armadillo repeat regions of p120 on the nuclear localisation of E-cad/CTF2 could be analysed in the future. Furthermore the identification of other nuclear binding partners for E-cad/CTF2 could provide further insight into the mechanism of its nuclear translocation.

Another possibility for the mechanism of nuclear transport of E-cad/CTF2 by p120 could be that p120 binding releases E-cad/CTF2 from sequestration in the cytosol by some other cytoplasmic binding partner. Overexpression of known E-cadherin-cytoplasmic-domain-binding proteins β-catenin and Hakai did not affect the nuclear localisation of E-cad/CTF2 (Figure 4.11). Hence unidentified binding partners of E-cad/CTF2 may be involved. It is interesting to note that E-cad/CTF2 behaves differently to N-cad/CTF2 when overexpressed in cells. When N-cad/CTF2 was overexpressed in neurons or neural crest cells, it localised predominantly in the nucleus (Shoval et al., 2007; Uemura et al., 2006a). In contrast, E-cad/CTF2 is predominantly localised in the cytoplasm and increased nuclear localisation is only observed upon overexpression of p120. It is possible that binding partners specific to N-cad/CTF2 or E-cad/CTF2 might play a role in the control of their subcellular localisation.

In summary, this chapter describes the nuclear localisation of E-cad/CTF2 and the role of p120 in its translocation. This raises the possibility of a role for E-cad/CTF2 in the nucleus and that E-cad/CTF2, as has been shown for Notch, may transmit cellular signals from the membrane to the nucleus.

CHAPTER 5

5 POST-TRANSLATIONAL MODIFICATION OF E-CAD/CTF2

5.1 Introduction

In this chapter, the post-translational modification of E-cad/CTF2 is described. During the biochemical fractionation studies described in chapter 4, it was observed that multiple higher molecular weight modifications of E-cad/CTF2 were frequently induced by coexpression of p120 (Figure 4.8A and 4.11). Here it is shown that E-cad/CTF2 is ubiquitinated and a potential E3-ubiquitin ligase for E-cad/CTF2 is identified. The role of ubiquitination in nuclear transport of E-cad/CTF2 is also investigated. Furthermore, it is also shown that E-cad/CTF2 is subject to phosphorylation and that this phosphorylation may also impact on the control of the nuclear localisation of E-cad/CTF2.

5.2 Ubiquitination of E-cad/CTF2

5.2.1 Ubiquitination of E-cad/CTF2 was identified using a pull-down assay with Nickel beads to precipitate His-tagged ubiquitin

Since p120 frequently induces multiple higher molecular weight modifications of E-cad/CTF2 (Figure 4.8A and 4.11), it was investigated whether they may represent ubiquitination of E-cad/CTF2. As described in section 1.6 (Introduction), ubiquitin is a highly conserved 8.5 kDa regulatory protein that can be covalently attached to lysine residues of proteins. Ubiquitin molecules can also covalently link to other ubiquitin monomers to form chains. This process is known as polyubiquitination and can result in the appearance of multiple slower running bands when the protein sample is examined by SDS-PAGE and Western blotting. Since a similar 'upsmear' of modification of E-cad/CTF2 was often observed when p120 is overexpressed, His-tagged ubiquitin was over-expressed together with E-cad/CTF2 and/or p120 in HEK293 cells and used in a pull-down assay to determine whether E-cad/CTF2 is ubiquitinated. His-tagged proteins can be precipitated from cell lysates using Nickel conjugated to agarose (DePace et

al., 1998; Kingsbury et al., 2000). Analysis of total HEK293 cell lysates showed that expression of His-tagged ubiquitin (His-Ub) induced modifications of E-cad/CTF2 that were of the expected size for the addition of 1 and 2 ubiquitin monomers (indicated by arrowheads, Figure 5.1, left panel). p120 expression also induced modification of E-cad/CTF2 of the expected size for the addition of one ubiquitin monomer (Figure 5.1, left panel, lane 4). The intensity of both higher molecular weight bands induced by His-Ub expression was increased by p120 expression (Figure 5.1, left panel, lane 5). The band of expected size for addition of one ubiquitin monomer was precipitated specifically by Nickel beads (indicated by arrow, Figure 5.1, right panel, lanes 3 and 5). This suggests that p120 induces ubiquitination of E-cad/CTF2. It should be noted that the extent of modifications varied between experiments (see section 5.6 for further discussion of this issue). In this experiment higher molecular weight modifications were not detected. However the possibility that the higher molecular weight modifications previously observed (Figure 4.8A and Figure 4.11) may be due to polyubiquitination or other ubiquitin-like modifiers cannot be ruled out.

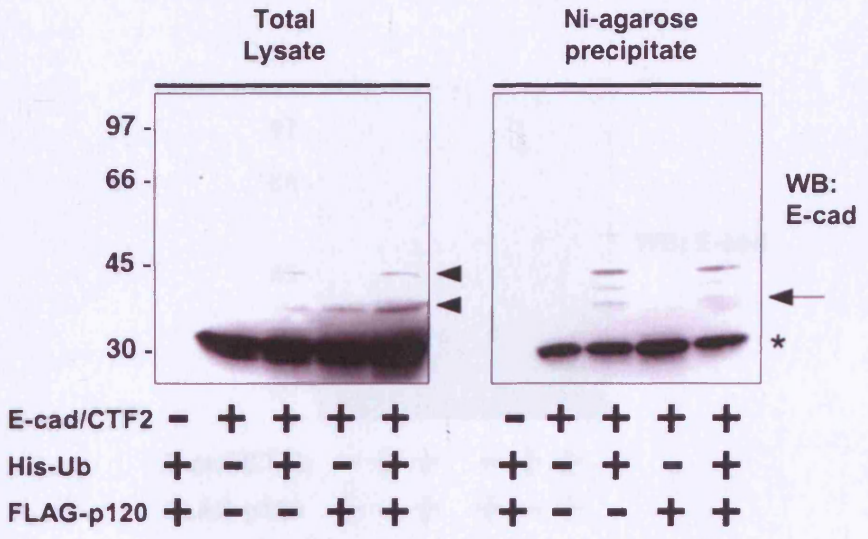


Figure 5.1 ***E-Cad/CTF2 is ubiquitinated.*** *Nickel (Ni)-agarose beads were incubated with lysates of HEK293 cells expressing E-cad/CTF2 together with FLAG-p120 and or His-tagged ubiquitin (His-Ub). The proteins bound to the beads were analysed by Western blotting with anti-E-cadherin antibody. *, High background was observed with this technique and although background was reduced by elution of the proteins bound to the Ni-agarose with imidazole, unmodified E-cadherin was non-specifically precipitated with the Ni-agarose. Arrowheads indicate modifications of E-cad/CTF2 of the molecular size expected for the addition of 1 or 2 ubiquitin monomers. An arrow indicates the band induced by expression of both p120 and His-Ub which is specifically precipitated by Ni-agarose.*

5.2.2 E-Cad/CTF2 and its modified bands are not stabilised by treatment with the proteasome inhibitor MG132

Ubiquitination of proteins has a variety of roles such as protein degradation in the proteasome, signalling, and intracellular protein transport including nuclear import (Geetha et al., 2005; Hochstrasser, 2004; Li et al., 2003; Mukhopadhyay and Riezman, 2007; Pickart, 2001; Shcherbik and Haines, 2004). Because coexpression of p120 and ubiquitin did not induce destabilisation of E-cad/CTF2 it seemed unlikely that ubiquitination targets E-cad/CTF2 for proteasomal degradation. To examine whether ubiquitination of E-cad/CTF2 leads to its degradation in the proteasome, HEK293 cells expressing E-cad/CTF2 with and without p120 were treated with a proteasome inhibitor, MG132 (Figure 5.2). If E-cad/CTF2 was targeted for degradation in the proteasome, treatment with MG132 should result in a stabilisation of E-cad/CTF2 and its modified bands. No significant stabilisation of E-cad/CTF2 or the modified bands was observed upon

incubation with MG132 (Figure 5.2). This indicates that it is unlikely that E-cad/CTF2 is being targeted for degradation in the proteasome.

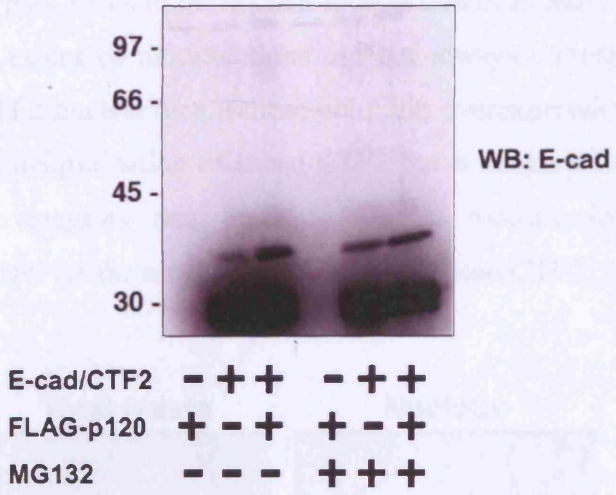


Figure 5.2 ***E-Cad/CTF2 is not stabilised by treatment with MG132.*** *HEK293 cells expressing E-cad/CTF2 together with FLAG-p120 were incubated with MG132 (20 μM) for 6 h. Total cell lysates were examined by Western blotting with anti-E-cadherin antibody.*

5.2.3 Ubiquitination of E-cad/CTF2 can enhance nuclear localisation of E-cad/CTF2

As p120 induces ubiquitination and nuclear translocation of E-cad/CTF2, it was investigated whether ubiquitination may play a role in its nuclear translocation. When HA-ubiquitin (HA-Ub) was coexpressed with E-cad/CTF2, slower migrating bands of E-cad/CTF2 were induced (Figure 5.3, left panel, fourth lane). Interestingly, coexpression of ubiquitin also enhanced nuclear localisation of E-cad/CTF2 (Figure 5.3, right panel, fourth lane). Coexpression of ubiquitin and p120 further enhanced the amounts of these modified bands and nuclear localisation of E-cad/CTF2 (Figure 5.3, left and right panels, sixth lane). A mutant form of ubiquitin (Ub (K 11, 27, 29, 48, 63 R) (HA-mut-Ub), which cannot form poly-ubiquitin chains, did not affect either nuclear translocation or modifications of E-cad/CTF2 (Figure 5.3, left and right panels, fifth and seventh lanes). Ubiquitinated forms of E-cad/CTF2 were not usually observed in the nuclear fraction (see Section 5.6 for further discussion). These data provide further supporting evidence that p120 potentiates ubiquitination of E-cad/CTF2.

The enhancement of multiple higher molecular weight modifications by HA-Ub, but not by HA-mut-Ub which cannot form polyubiquitin chains, suggests that E-cad/CTF2 can be polyubiquitinated. The data in Figure 5.3 also suggest that ubiquitination can play a role in the nuclear localisation of E-cad/CTF2. It should be noted that the extent of modifications did not always correspond with the extent of E-cad/CTF2 nuclear localisation; ubiquitin overexpression has a strong effect on inducing ubiquitination of E-cad/CTF2 but a weaker effect on nuclear localisation. This suggests that there is also a modification-independent molecular mechanism for the nuclear localisation of E-cad/CTF2.

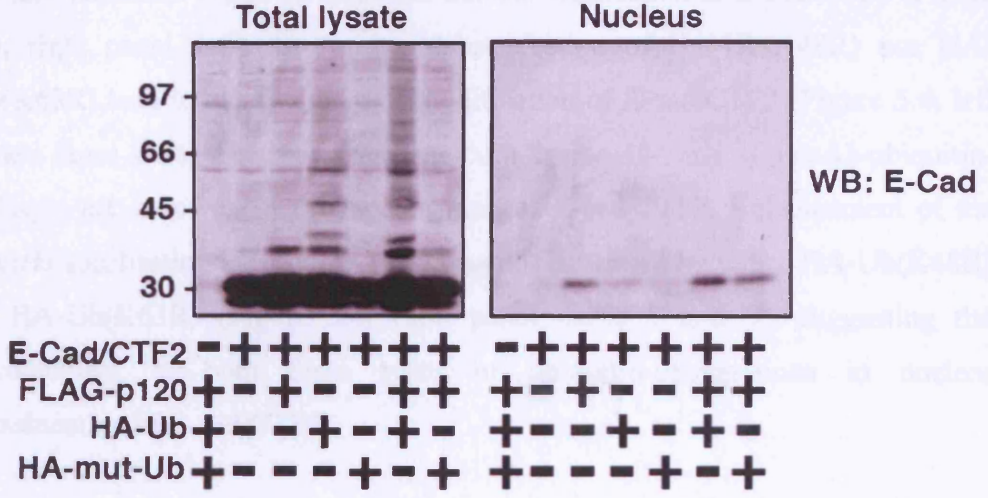


Figure 5.3 ***Overexpression of ubiquitin enhances the nuclear localisation and modification of E-cad/CTF2.*** *HEK293 cells were transfected with E-cad/CTF2 together with FLAG-p120 and/or HA-ubiquitin (HA-Ub) or HA-ubiquitin(K11, 27, 29, 48, 63 R) (HA-mut-Ub). Total and nuclear lysates were examined by Western blotting with anti-E-cadherin antibody.*

5.2.4 Lysine 48- and lysine 63- conjugated Ubiquitin are both involved in the modification and nuclear translocation of E-cad/CTF2

As described in section 1.6.2 (Introduction), the type of ubiquitin linkages used in the formation of polyubiquitin chains, can affect its molecular function (Sun and Chen, 2004; Varadan et al., 2004). It is generally thought that lysine 48-linked polyubiquitin chains target proteins to the proteasome for degradation whereas lysine 63-linked polyubiquitin chains have other signalling roles (Sun

and Chen, 2004). However it is now clear that lysine 48-linked polyubiquitin chains have other roles besides the targeting of proteins for proteasomal degradation (Hochstrasser, 2004). To determine whether E-cad/CTF2 is modified by lysine-48- or lysine-63-conjugated polyubiquitin chains, two further ubiquitin mutants were used: HA-Ub(K48R) in which lysine residue 48 of ubiquitin is mutated to arginine preventing it from forming lysine-48-linked chains; and HA-Ub(K63R) in which lysine residue 63 of ubiquitin is mutated to arginine preventing it from forming lysine-63-linked chains. As is also shown in Figure 5.3, cotransfection of wild type HA-Ub led to enhancement of modification of E-cad/CTF2 (Figure 5.4, left panel, lanes 4 and 5). Cotransfection of wild type HA-Ub also enhanced the p120-mediated-nuclear localisation of E-cad/CTF2 (Figure 5.4, right panel, lane 5). Neither cotransfection of HA-Ub(K48R) nor HA-Ub(K63R) lead to enhancement of modification of E-cad/CTF2 (Figure 5.4, left panel, lanes 6-9). This suggests that both lysine-48- and lysine-63-ubiquitin-linkages are involved in the modification of E-cad/CTF2. Enhancement of the nuclear localisation induced by p120 was not enhanced by either HA-Ub(K48R) or HA-Ub(K63R) (Figure 5.4, right panel, lanes 7 and 9), suggesting the involvement of both these types of ubiquitin-conjugations in nuclear translocation of E-cad/CTF2.

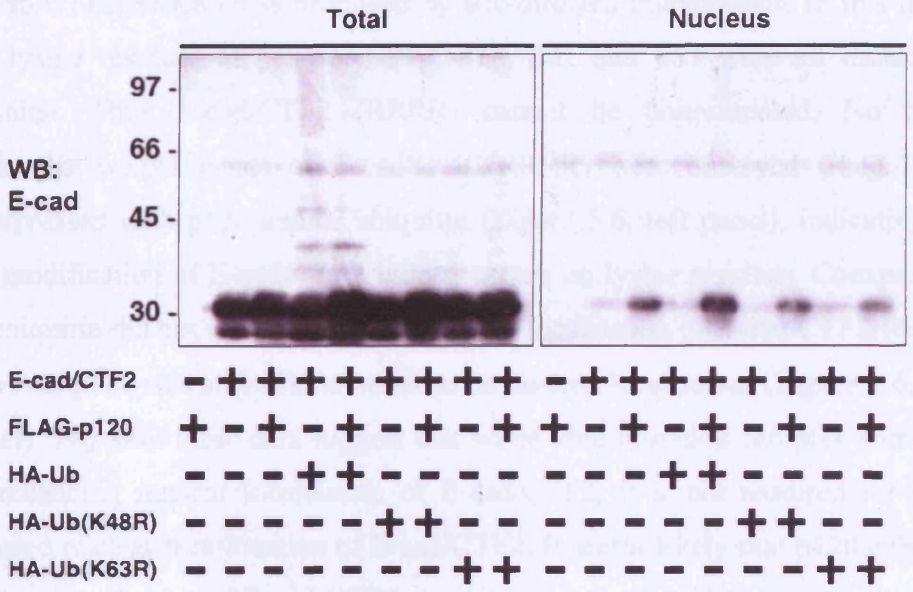


Figure 5.4 ***Lysine-48- and Lysine-63-conjugated ubiquitination are both involved in the modification and nuclear translocation of E-cad/CTF2.*** *HEK293 cells were transfected with E-cad/CTF2 together with FLAG-p120 and/or HA-ubiquitin (HA-Ub) or HA-Ub(K48R) or HA-Ub(K63R). Total and nuclear lysates were examined by Western blotting with anti-E-cadherin antibody*

5.2.5 Ubiquitination is not the only mechanism for the p120-induced nuclear translocation of E-cad/CTF2

The role of ubiquitination in the nuclear localisation of E-cad/CTF2 was further examined by mutating all the lysine residues within E-cad/CTF2 to arginine. The location of the four lysine residues in E-cad/CTF2 that are potenital targets for ubiquitination are shown in Figure 5.5.

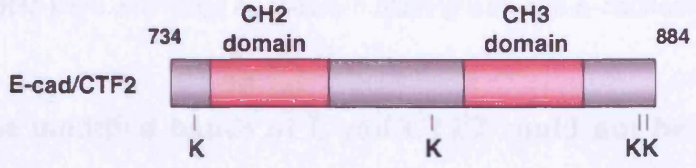


Figure 5.5 ***Location of lysine residues in E-cad/CTF2.*** *E-Cad/CTF2 contains four lysine residues (K) that are potential targets for ubiquitination.*

E-Cad/CTF2(RRRR) was produced by site-directed mutagenesis. In this mutant the lysine residues at positions 740, 818, 861 and 863 were all mutated to arginine. Thus E-cad/CTF2 (RRRR) cannot be ubiquitinated. No higher molecular weight bands of E-cad/CTF2(RRRR) were observed when it was coexpressed with p120 and/or ubiquitin (Figure 5.6, left panel), indicating that the modification of E-cad/CTF2 indeed occurs on lysine residues. Coexpression of ubiquitin did not induce enhanced nuclear localisation of E-cad/CTF2(RRRR), however p120 was still able to enhance its nuclear localisation (Figure 5.6, right panel). Together these data suggest that while ubiquitination can play some role in enhancing nuclear localisation of E-cad/CTF2, it is not required for p120-induced nuclear translocation of E-cad/CTF2. It seems likely that p120 enhances nuclear localisation of E-cad/CTF2 via a ubiquitin-independent pathway.

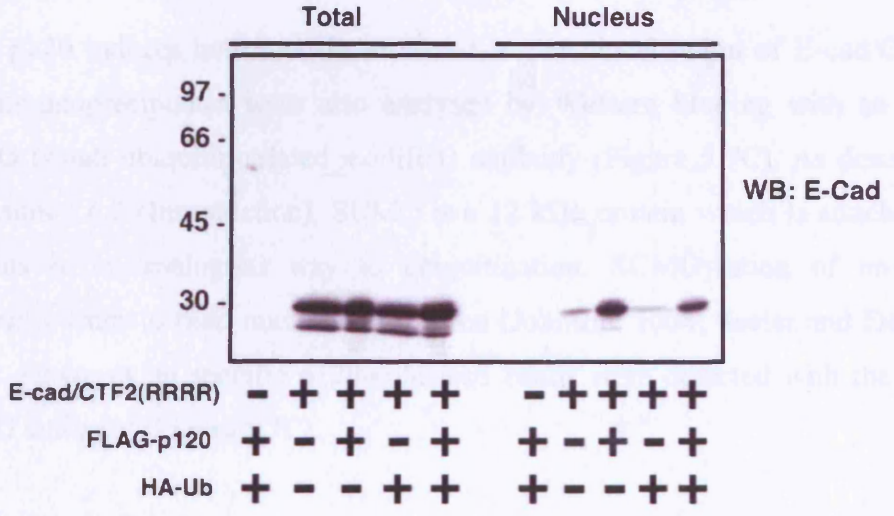


Figure 5.6 ***p120 enhances nuclear localisation of E-cad/CTF2(RRRR) in which all lysine residues were mutated to arginine.*** *HEK293 cells were transfected with E-cad/CTF2(RRRR) together with FLAG-p120 and/or HA-ubiquitin (HA-Ub). Total and nuclear lysates were examined by Western blotting with anti-E-cadherin antibody.*

5.2.6 The modified bands of E-cad/CTF2 could not be recognised by Western blotting with anti-ubiquitin or anti-SUMO antibodies.

As shown in Figures 4.8A and 4.11, coexpression of p120 frequently enduces higher molecular weight modifications of E-cad/CTF2, that are detected in total lysates even in the absence of overexpressed ubiquitin. It was next examined whether these bands, produced in the absence of overexpressed ubiquitin, could

be detected by Western blotting with an anti-ubiquitin antibody. Lysates from HEK293 cells transfected with E-cad/CTF2 and p120 were immunoprecipitated with anti-E-cadherin antibody and the immunoprecipitates were then examined by Western blotting. Western blotting with anti-E-cadherin antibody showed that the bands representing modification were immunoprecipitated and that p120 enhanced the appearance of the modified bands (Figure 5.7A). When imunoprecipitates were analysed with anti-Ubiquitin (Ub) antibody, although some background bands appeared in all lanes, no bands could be detected that were specifically enhanced by p120 expression (Figure 5.7B). This does not rule out the possibility that the modifications are ubiquitination since the ubiquitin antibody may have a lower sensitivity than the E-cadherin antibody. Indeed in many cases overexpression of ubiquitin is used to detect ubiquitination of proteins (Chung et al., 2004; Matsuzaki et al., 2003).

Since p120 induces both modification and nuclear localisation of E-cad/CTF2, the immunoprecipitates were also analysed by Western blotting with an anti-SUMO (small ubiquitin-related modifier) antibody (Figure 5.7C). As described in section 1.6.2 (Introduction), SUMO is a 12 kDa protein which is attached to proteins in an analogous way to ubiquitination. SUMOylation of proteins frequently leads to their nuclear localisation (Johnson, 2004; Seeler and Dejean, 2003). However no specific p120-enhanced bands were detected with the anti-SUMO antibody (Figure 5.7C).

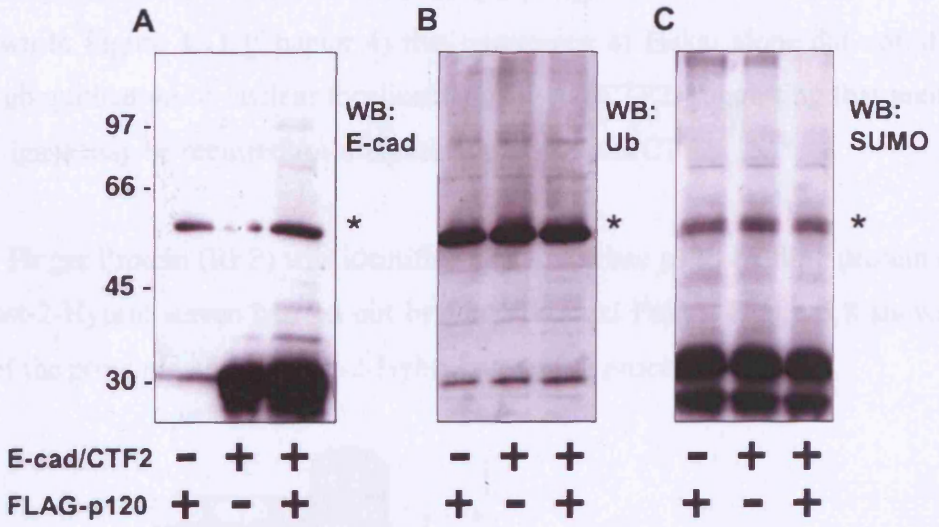


Figure 5.7 ***The higher molecular weight modified bands of E-cad/CTF2 were not recognised with anti-Ubiquitin (Ub) or anti-SUMO antibodies.*** *Lysates of HEK293 cells expressing E-cad/CTF2 with and without FLAG-p120 were immunoprecipitated with anti-E-cadherin antibody, followed by Western blotting with (A) anti-E-cadherin, (B) anti-Ubiquitin (Ub), and (C) anti-SUMO antibodies. *, heavy chain of IgG.*

5.3 Characterisation of a novel p120-binding protein, Ret Finger Protein, which is a candidate as a novel E3-ligase for E-cad/CTF2

5.3.1 Ret Finger Protein, a novel binding partner of p120

In sections 5.2 and 5.3, E-cad/CTF2 was shown to be ubiquitinated and this ubiquitination was also shown to have a role in its nuclear localisation of E-cad/CTF2. As described in section 1.6.1 (Introduction), the final step of the ubiquitin-conjugation pathway is carried out by an E3-ubiquitin-ligase. E3-ubiquitin-ligases are usually specific for the protein substrate which receives the ubiquitin conjugation (Laney and Hochstrasser, 1999). Therefore it is likely that p120 recruits an E3-ligase which can ubiquitinate E-cad/CTF2. As described in section 1.4.4 (Introduction) Hakai is an E3-ubiquitin-ligase for full length E-cadherin and is involved in down-regulation of E-cadherin at cell-cell contacts, by ubiquitinating E-cadherin and triggering its endocytosis (Fujita et al., 2002; Tricaud et al., 2005). However Hakai and p120 were shown to compete for binding to the intracellular domain of E-cadherin (Fujita et al., 2002), and so it is

unlikely that Hakai could be the E3-ubiquitin ligase in this case. Indeed, it was shown in Figure 4.11 (Chapter 4) that expression of Hakai alone did not affect the ubiquitination or nuclear localisation of E-cad/CTF2, suggesting that another E3-ligase may be required for ubiquitination of E-cad/CTF2.

Ret Finger Protein (RFP) was identified as a candidate p120-binding protein in a Yeast-2-Hybrid screen carried out by Dr. Yasuyuki Fujita. Figure 5.8 shows in brief the principle of the Yeast-2-Hybrid screening procedure.

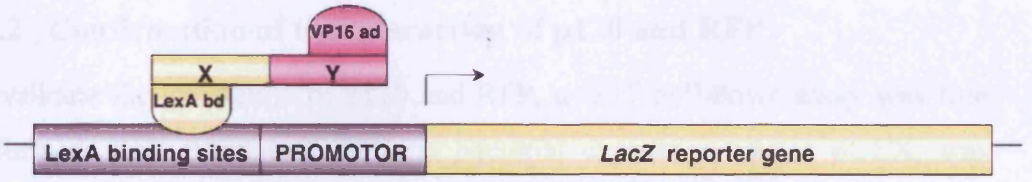


Figure 5.8 ***Yeast-2-Hybrid screening.*** *The bait gene (X) is fused to the LexA DNA binding domain (bd). Prey genes are fused to the VP16 activation domain (ad). This bait construct is transfected into yeast along with a LacZ reporter construct. The LexA bd binds to LexA binding sites in the minimal promotor region of the LacZ reporter construct. When the gene products of X and Y interact, the VP16 activation domain is brought into proximity of the promotor region and transcription from LacZ is activated (Proof of concept described in (Fields and Song, 1989)).*

In this screen a Hollenberg (mouse embryo) cDNA library was screened using the open reading frame of mouse p120 protein isoform B as the 'bait' gene. RFP was identified as a potential binding partner. As described in section 1.6.3 (Introduction), RFP is a protein whose biological function is not fully characterised. It has been shown to regulate transcription and to shuttle in and out of the nucleus.

RFP contains three major protein interaction domains: a RING finger domain, a B-box domain, and a coiled-coil protein interaction domain (Figure 5.9). It is a member of the TRIM/RBCC family of proteins which contain all three motifs depicted in Figure 5.9. The yeast-2-hybrid screen showed that the coiled-coil region is responsible for the binding to p120. As described in section 1.6.1, many E3-ubiquitin-ligases including Hakai contain RING finger domains, which are vital to their function. Thus, RFP is a possible candidate as an E3-ubiquitin-ligase for E-cad/CTF2. Indeed, several TRIM/RBCC proteins have been shown to have E3-ubiquitin ligase activity (Meroni and Diez-Roux, 2005).

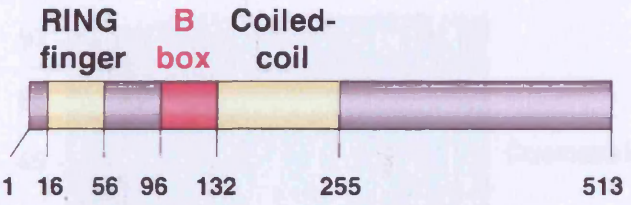


Figure 5.9 ***Domain structure of Ref Finger Protein (RFP).*** *RFP contains a RING finger domain, a B box domain and a Coiled-coil region.*

5.3.2 Confirmation of the interaction of p120 and RFP

To validate the interaction of p120 and RFP, a GST pull-down assay was first performed. RFP fused to GST in a bacterial expression vector pGEX, was constructed (see table 2.5, section 2.1.11 Materials and Methods, for details) and expressed in *E.coli*. Although good expression levels of GST-RFP were achieved, GST-RFP could not be solubilised in either Triton-X-100 (Figure 5.10A) or RIPA (Figure 5.10B) lysis buffers. Therefore GST-RFP could not be recovered in the supernatant of the clarified lysate or following precipitation with glutathione-sepharose (Figure 5.10 A and B). Bacterially expressed recombinant proteins can become accumulated in inclusion bodies (Hoffmann et al., 2001). Hence to solubilise GST-RFP, inclusion bodies were solubilised in mildly denaturing conditions. Following clarification, the soluble fraction was dialysed against a neutral pH buffer containing a reducing agent to encourage correct disulfide bond formation. This was then followed by a second dialysis step to remove excess reducing agent. GST-RFP could then be precipitated with glutathione-sepharose, and its concentration was determined (Figure 5.11).

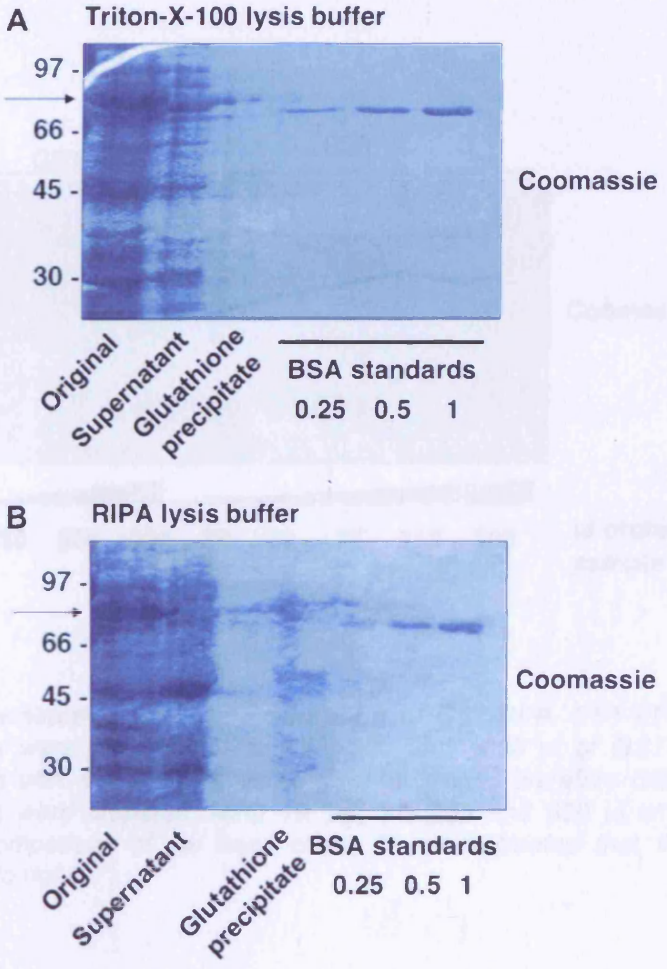


Figure 5.10 ***Insolubility of bacterially expressed GST-RFP.*** *(A and B) E. coli were transformed with pGEX-RFP and cultured as described in section 2.3.4 (Materials and Methods). After lysis in (A)Triton X-100 lysis buffer and (B) RIPA buffer, lysates were sonicated and centrifuged. Aliquots of the original (before centrifugation), supernatant and glutathione-sepharose-beads precipitate were analysed on an SDS-PAGE gel and proteins were visualized with Coomassie protein staining. Expression of GST-RFP was observed in the original (indicated with arrows), but was not solubilised and therefore not found in the supernatant or glutathione precipitate.*

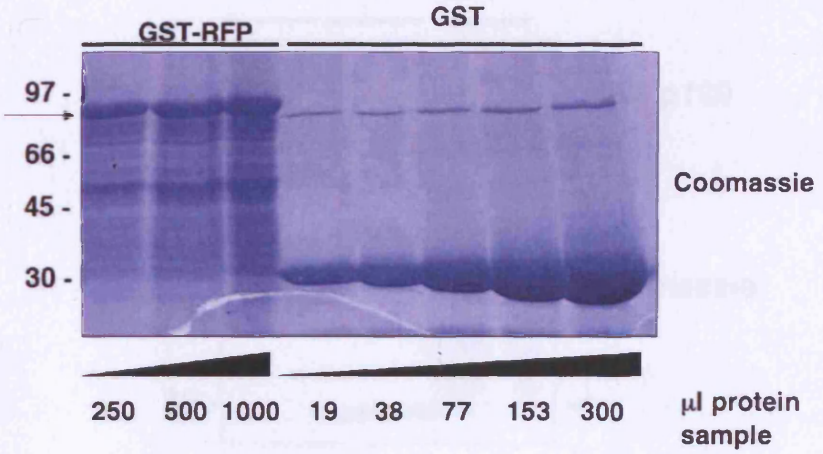


Figure 5.11 ***Determination of the concentration of GST-RFP.*** *GST-RFP-conjugated sepharose beads were prepared using 250, 500 and 1000 µl of GST-RFP protein sample (indicated with arrow). GST protein is at 1.3 µg/µl therefore GST-conjugated sepharose beads were prepared using 19, 33, 77, 153 and 300 µl of GST protein sample. From comparison of the band sizes, it was estimated that the GST-RFP concentration is 25 ng/µl.*

Next the interaction between p120 and RFP was examined by GST pull-down assay. GST-RFP specifically precipitated p120 from lysates of HEK293 cells (Figure 5.12). To further confirm the interaction between p120 and RFP, an endogenous coimmunoprecipitation was performed. Endogenous p120 from lysates of HEK293 cells was precipitated by anti-RFP antibody but not by control IgG (Figure 5.13). Taken together, these data indicate a specific interaction between p120 and RFP.

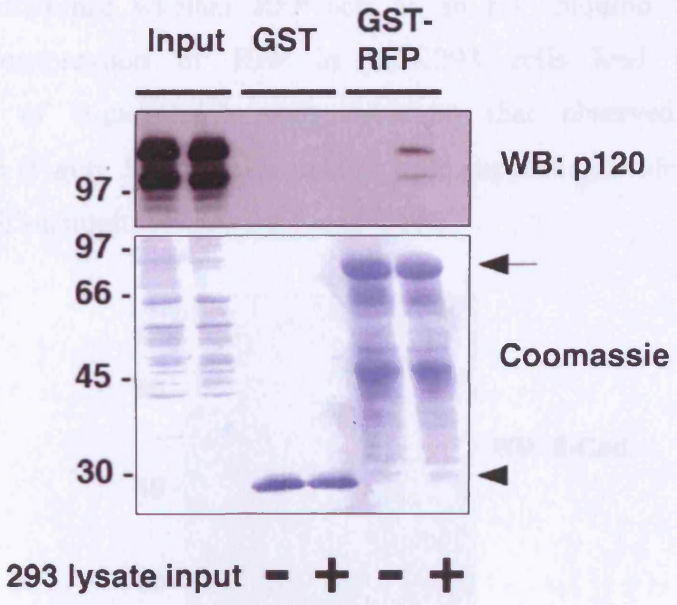


Figure 5.12 ***p120 interacts with RFP in a GST pull-down assay.*** *Beads coupled to GST or GST-RFP were incubated with lysates of HEK293 cells. The proteins bound to the beads were analysed by Coomassie staining and Western blotting with anti-p120 antibody. The arrow and arrowhead indicate the positions of GST-RFP and GST, respectively.*

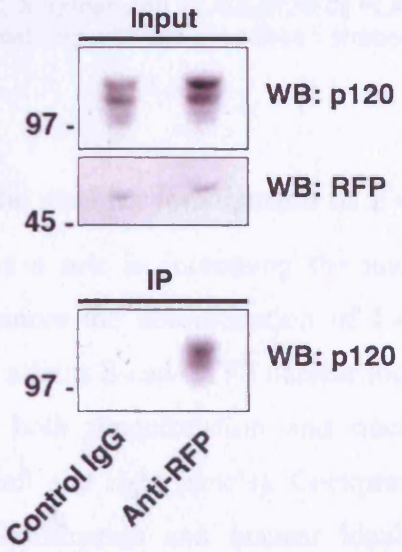


Figure 5.13 ***p120 interacts with RFP in an endogenous immunoprecipitation.*** *Lysates of HEK293 cells were immunoprecipitated by anti-RFP antibody or control IgG. Inputs were examined by Western blotting with anti-p120 and anti-RFP antibodies. Immunoprecipitates (IP) were examined by Western blotting with anti-p120 antibody. It was not possible to examine immunoprecipitates with anti-RFP antibody since the heavy chain of IgG is of the same molecular size as RFP.*

5.3.3 RFP enhances modification of E-cad/CTF2

It was next examined whether RFP acts as an E3 ubiquitin ligase for E-cad/CTF2. Coexpression of RFP in HEK293 cells lead to enhanced ubiquitination of E-cad/CTF2, comparable to that observed with p120 overexpression (Figure 5.14). These data provide supporting evidence that RFP may act as an E3-ubiquitin-ligase for E-cad/CTF2.

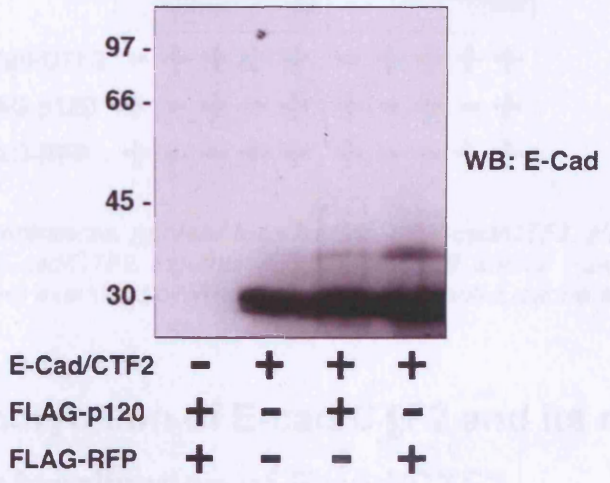


Figure 5.14 ***RFP enhances ubiquitination of E-cad/CTF2.*** *HEK293 cells were transfected with E-cad/CTF2 together with FLAG-p120 or FLAG-RFP. Total cell lysates were examined by Western blotting with anti-E-cadherin antibody.*

5.3.4 RFP enhances the nuclear localisation of E-cad/CTF2

Since ubiquitination plays a role in increasing the nuclear localisation of E-cad/CTF2, and RFP enhances the ubiquitination of E-cad/CTF2, it was next investigated whether RFP affects E-cad/CTF2 nuclear localisation. Expression of p120 or RFP increased both ubiquitination and nuclear localisation of E-cad/CTF2 (Figure 5.15, left and right panels). Coexpression of p120 and RFP further enhanced the ubiquitination and nuclear localisation of E-cad/CTF2 (Figure 5.15, left and right panels). These data provide further support for a role of RFP in the ubiquitination of E-cad/CTF2 and for the role of ubiquitination in nuclear localisation of E-cad/CTF2.

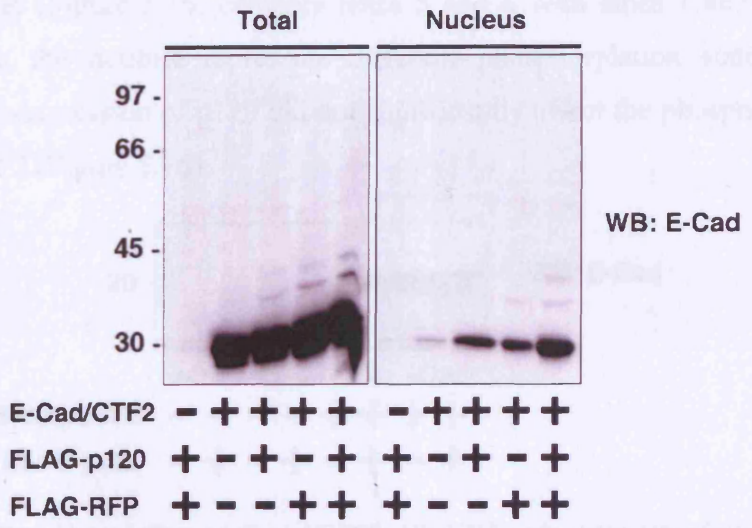


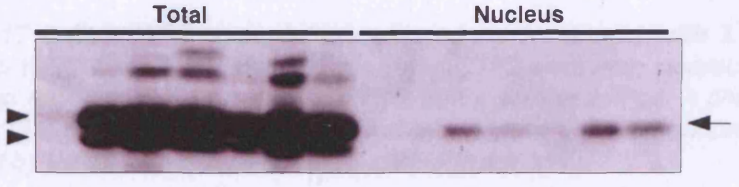
Figure 5.15 ***RFP enhances nuclear localisation of E-cad/CTF2.*** *HEK293 cells were transfected with E-cad/CTF2 together with FLAG-p120 and/or FLAG-RFP.Total and nuclear lysates were examined by Western blotting with anti-E-cadherin antibody.*

5.4 Phosphorylation of E-cad/CTF2 and its role in the nuclear localisation of E-cad/CTF2

5.4.1 E-cad/CTF2 is phosphorylated

It was observed in several experiments that when expressed in HEK293 cells, E-cad/CTF2 appears as a doublet when examined by Western blotting. To demonstrate this, part of Figure 5.4 is shown repeated below[1], with the doublet of E-cad/CTF2 indicated with arrowheads. Interestingly, only the upper band of the doublet is found in the nuclear fraction (indicated with an arrow, part of Figure 5.4, repeated below[1]). It was investigated whether these bands may represent different phosphorylation states of E-cad/CTF2. To test whether phosphorylation affects the doublet bands of E-cad/CTF2, HEK293 cells expressing E-cad/CTF2 were first treated with Okadaic Acid (OA), an inhibitor of the protein phosphatases PP1 and PP2A. Treatment with OA led to a loss of the lower band

[1]Part of Figure 5.4



(repeat)

of the doublet (Figure 5.16, compare lanes 5 and 6 with lanes 7 and 8). This suggests that the doublet represents different phosphorylation states of E-cad/CTF2. Coexpression of p120 did not significantly affect the phosphorylation of E-cad/CTF2 (Figure 5.16).

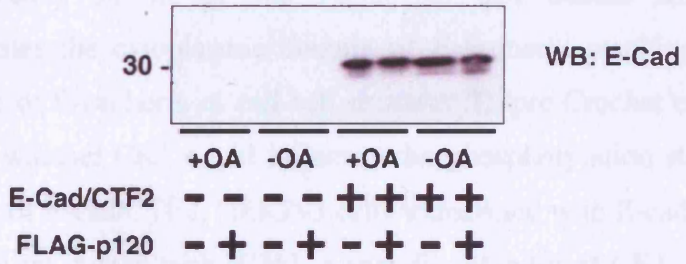


Figure 5.16 ***Phosphorylation of E-cad/CTF2.*** *HEK293 cells were transfected with E-cad/CTF2 and/or FLAG-p120 and then treated with Okadaic acid (OA) (3.13 ng/ml) for 4 hr.Total cell lysates were examined by Western blotting with anti-E-cadherin antibody.*

To further examine the phosphorylation of E-cad/CTF2, lysates of HEK293 cells expressing E-cad/CTF2 were immunoprecipitated with anti-E-cadherin antibody and then treated with λ phosphatase. Immunoprecipitates treated with λ phosphatase showed a shift from the upper band of the doublet to the lower band (Figure 5.17). This provides further evidence that E-cad/CTF2 is phosphorylated. Together with previous data showing that only the upper band of the doublet is found in the nucleus (Figure 5.4), these data raise the interesting possibility that phosphorylation may also regulate the nuclear translocation of E-cad/CTF2.

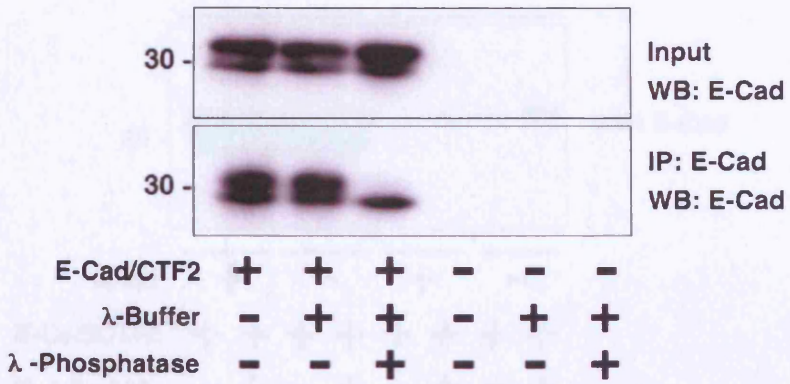


Figure 5.17 ***E-Cad/CTF2 is dephosphorylated by treatment with λ phosphatase.*** *Lysates of HEK293 cells transfected with E-cad/CTF2 were immunoprecipitated by anti-E-cadherin antibody and then treated with λ buffer with or without λ phosphatase (100 units) for 30 minutes at 30 °C. Cell lysates (Input) and immunoprecipitates were examined by Western blotting with anti-E-cadherin antibody.*

5.4.2 Casein Kinase 1 can phosphorylate E-cad/CTF2 and influence its nuclear localisation

It was recently shown by the Fujita lab that Casein Kinase 1 (CK1) phosphorylates the cytoplasmic domain of E-cadherin resulting in the down-regulatation of E-cadherin at cell-cell contacts (Dupre-Crochet et al., 2007). To investigate whether CK1 could influence the phosphorylation state and nuclear localisation of E-cad/CTF2, HEK293 cells transfected with E-cad/CTF2 together with p120 were treated with IC261, a specific inhibitor of CK1. Treatment with IC261 led to a mild increase in the lower, dephosphorylated form of the doublet (Figure 5.18), suggesting that treatment with CK1 inhibitor increases the amount of the dephosphorylated form of E-cad/CTF2. This suggests that CK1 may be involved in the phosphorylation of E-cad/CTF2, but that other kinases are also involved. Treatment with IC261 also reduced the amount of E-cad/CTF2 found in the nuclear fraction (Figure 5.18). This may suggest that the loss of the phosphorylated form of E-cad/CTF2 in these conditions leads to less nuclear translocation. However, the effect of IC261 on nuclear localisation is greater than the observed effect on the doublet, possibly suggesting that other proteins phosphorylated by CK1 could also affect the nuclear localisation of E-cad/CTF2.

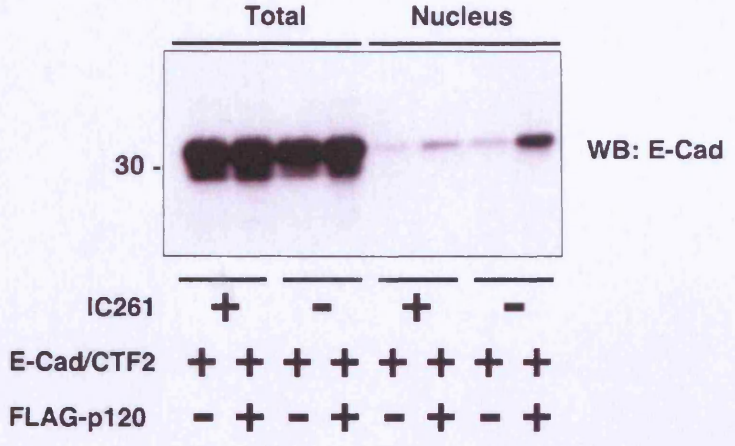


Figure 5.18 ***Treatment with the CK1 inhibitor leads to dephosphorylation of E-cad/CTF2 and decreases its nuclear localisation.*** *HEK293 cells transfected with E-cad/CTF2 with or without p120 were treated with IC261 (5 μg/ml) for 4 h. Total and nuclear lysates were examined by Western blotting with anti-E-cadherin antibody.*

To further investigate the effect of CK1 on the phosphorylation of E-cad/CTF2, myc-tagged constructs of two isoforms of CK1 were made (see table 2.3, section 2.1.11, Materials and Methods, for details). As described in section 1.4.4 (Introduction), there are at least seven isoforms of CK1 found in human cells, but CK1α and CK1ε are the most frequently expressed in tissues (Knippschild et al., 2005). Thus these two isoforms were used in expression studies. Phosphorylation often acts as a priming event for ubiquitination of proteins, for example CK1 phosphorylation of β-catenin induces ubiquitination of β-catenin by the APC complex (Polakis, 2002). Since E-cad/CTF2 is subject to both phosphorylation and ubiquitination, and both of these post-translational modifications have a positive effect on the nuclear translocation of E-cad/CTF2, the effect of expressing CK1α and CK1ε on both ubiquitination and nuclear translocation of E-cad/CTF2 was examined. Coexpression of CK1α or CK1ε lead to a mild upregulation of both ubiquitination and nuclear translocation of E-cad/CTF2 (Figure 5.19). However, it was difficult to see a clear effect on the doublet bands (Figure 5.19). Taken together, these data suggest that CK1 can phosphorylate E-cad/CTF2 which enhances both its ubiquitination and nuclear localisation. However it seems likely that other kinases are also involved in the phosphorylation of E-cad/CTF2.

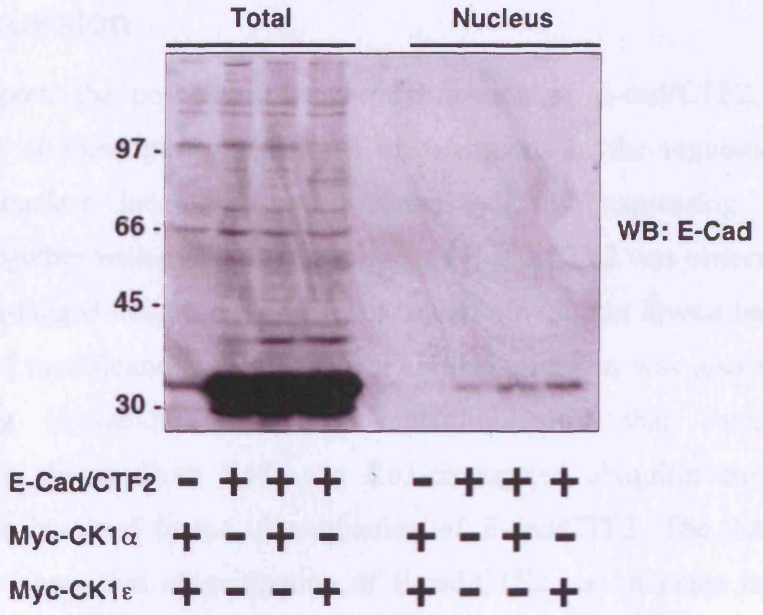


Figure 5.19 ***CK1 enhances ubiquitination and nuclear localisation of E-cad/CTF2.*** *HEK293 cells were transfected with E-cad/CTF2 together with CK1α and CK1ε. Total and nuclear lysates were examined by Western blotting with anti-E-cadherin antibody.*

5.5 Discussion

In this chapter, the post-translational modification of E-cad/CTF2, and the involvement of these post-translational modifications in the regulation of E-cad/CTF2 nuclear localisation were described. By expressing ubiquitin constructs together with p120, ubiquitination of E-cad/CTF2 was observed. Pull-down of His-tagged ubiquitin with Nickel beads showed that lowest band in the 'upsmear' of modifications is ubiquitin. Polyubiquitination was also shown to occur using HA-ubiquitin and HA-mutant-ubiquitin that cannot form polyubiquitin chains. Both K48- and K63-conjugated ubiquitin chains were shown to be involved in the ubiquitination of E-cad/CTF2. The data in this chapter also show that ubiquitination of E-cad/CTF2 can enhance its nuclear localisation. RFP has been identified as a RING-finger-domain-containing protein which binds p120. Expression of RFP also enhances ubiquitination and nuclear localisation of E-cad/CTF2 and is therefore a potential candidate as an E3-ubiquitin ligase for E-cad/CTF2. Finally, it was shown that E-cad/CTF2 is phosphorylated and only the more phosphorylated form is found in the nucleus. CK1 may play a role in the phosphorylation of E-cad/CTF2 and its nuclear localisation.

As noted in chapter 4, the amount of ubiquitination varies between experiments. Ubiquitinated forms of E-cad/CTF2 are sometimes observed in the nuclear fraction (Figure 5.16), but in most experiments, ubiquitinated E-cad/CTF2 cannot be observed in the nuclear fraction. It is highly likely that despite the addition of the *N*-ethylmaleimide to inhibit de-ubiquitination, the de-ubiquitination of E-cad/CTF2 occurs very rapidly upon cell lysis. Indeed it was noted that the ubiquitinated bands were frequently lost when the lysates were stored for a longer period. As the process of nuclear fractionation takes longer than the preparation of total cell lysates, this could be one explanation for the loss of ubiquitinated forms of E-cad/CTF2 from the nuclear fraction. However it is also possible that E-cad/CTF2 becomes de-ubiquitinated when it enters the nucleus of cells.

Despite several attempts, it was not possible to detect the ubquitin bands by Western blotting with anti-ubiquitin antibody, although ubiquitination could be observed upon expression of ubiquitin constructs. While the expression studies show that E-cad/CTF2 is ubiquitinated, it cannot be excluded that other ubiquitin-like modifiers are involved in the modification of E-cad/CTF2. Mass spectrometry could be used to formally identify these bands. However it was not possible to detect the modified bands of E-cad/CTF2 on an SDS-PAGE gel using either sensitive coomassie, SYPRO® Ruby or silver staining, even when > 5 x 10^6 cells were used per experiment. Thus it was not possible to isolate the modified bands for identification by mass spectrometry. The inability to detect the bands on an SDS-PAGE gel by any of these methods suggests that the level of E-cad/CTF2 protein is not very high, or that the levels of ubiquitination is low, which could explain why it was not possible to detect ubiquitination with anti-ubiquitin antibody.

Induction of ubiquitination by expression of p120, RFP or ubiquitin did not induce destablisation of E-cad/CTF2. Furthermore, the stability of E-cad/CTF2 or its ubiquitinated forms were not affected by inhibition of the proteasome. These data suggest that it is unlikely that ubiquitination of E-cad/CTF2 is involved in the targeting of E-cad/CTF2 for degradation in the proteasome. Ubiquitination has many other roles in signalling and intracellular transport, including nuclear import (Geetha et al., 2005; Hochstrasser, 2004; Li et al., 2003; Mukhopadhyay and Riezman, 2007; Pickart, 2001; Shcherbik and Haines, 2004). As described in section 1.6.2, different ubiquitin linkages form structurally different polyubiquitin chains and are thought to have different roles (Sun and Chen, 2004; Varadan et al., 2004). Lysine-48-linked polyubiquitin chains have classically been thought to target proteins for degradation, whereas lysine-63-linked polyubiquitin chains have roles in signalling and intracellular transport of proteins (Sun and Chen, 2004). Lysine-63-linked chains have a more open comformation with more hydrophobic surfaces exposed, which may provide an interface for the interaction with other proteins. Indeed a recent study showed that lysine-63-conjugated polyubiquitination induces the nuclear import of neurotrophin receptor interacting factor (NRIF) (Geetha et al., 2005). In this chapter, the results using mutant ubiquitin constructs (K48R and K63R) suggest

that both lysine-48- and lysine-63- linked ubiquitin chains are involved in the modification and nuclear import of E-cad/CTF2. It is not clear whether this represents polyubiquitin chains with mixed linkages or whether there maybe more than one polyubiquitin chain, each containing a single type of linkage, conjugated to the different lysine residues within E-cad/CTF2. As described in 1.6.2, lysine-48-linked chains have also been shown to have roles other than targeting proteins to the proteasome (Hochstrasser, 2004). It appears that lysine-48-linked chains as well as lysine-63-linked chains are involved in regulating the nuclear localisation of E-cad/CTF2, however it cannot be ruled out that other types of ubiquitin linkages may also be involved.

While ubiquitination enhances the nuclear localisation of E-cad/CTF2, studies of the nuclear localisation of E-cad/CTF2(RRRR), in which all of the lysine residues were mutated to arginine thus preventing ubiquination, suggest that p120 can induce nuclear transport of E-cad/CTF2 by a ubiquitin-independent mechanism. Nuclear localisation of E-cad/CTF2(RRRR) was not changed upon coexpression of ubiquitin. However, p120 induced nuclear localisation of E-cad/CTF2(RRRR), indicating a ubiquitin-independent mechanism. This mechanism may involve either direct nuclear import via the interaction with p120 or the release of E-cad/CTF2 from sequestration from another cellular location (see section 4.6 for further discussion). How ubiquitination enhances the nuclear localisation of E-cad/CTF2 is also still unclear. Addition of ubiquitin to the E2-conjugating enzymes UbcM2, UbcH6 and UBE2E2 has been shown to enhance their nuclear translocation. The addition of ubiquitin to these proteins enhances their binding to importin-11, which promotes their nuclear localisation (Plafker et al., 2004). However, as described in section 1.6.1, this linkage is a thioester linkage, which results in the 'ubiquitin charging' of the E2-conjugating enzymes prior to the transfer of ubiquitin to the protein target by E3-ubiquitin-ligases. This thioester linkage is different to the linkages between ubiquitin monomers in a polyubiquitin chain or between ubiquitin and substrate proteins. It is not clear whether polyubiquination or monoubiquitination of proteins can enhance their binding to importins. The identification of binding proteins specfic to the ubiquitinated forms of E-cad/CTF2 could provide further insight into the mechanism of nuclear transport.

Since p120 induces ubiquitination of E-cad/CTF2, it is likely that it recruits an E3-ubiquitin-ligase in order to facilitate the ubiquitination of E-cad/CTF2. RFP was identified as a p120-binding protein which contains a RING-finger domain, and which also enhances the ubiquitination and nuclear localisation of E-cad/CTF2. It is therefore a good candidate as the E3-ligase for E-cad/CTF2. To formally establish whether RFP has E3-ubiquitin ligase activity for E-cad/CTF2, an *in vitro* ubiquitination assay should be performed. Knocking down RFP using RNAi would also provide further insight into its role in enhancing the ubiquitination and nuclear localisation of E-cad/CTF2.

E-Cad/CTF2 was also shown to be phosphorylated and interestingly, ony the more phosphorylated form was observed in the nuclear fraction. Data shown in Figure 4.2 indicate that endogenously produced E-cad/CTF2 is mostly found in the more phosphorylated form, and therefore has the potential to enter the nucleus. CK1 was shown to play a role in the phosphorylation of E-cad/CTF2 and in the regulation of its ubiquitination and nuclear localisation, however it is not the only kinase involved. Identification of other kinases that phosphorylate E-cad/CTF2 could provide more insight into the regulation of E-cad/CTF2 nuclear localisation. As described in sections 1.4.4, E-cadherin is known to be phosphorylated by both serine/threonine and tyrosine kinases. Whether the observed phosphorylation of E-cad/CTF2 is due to phosphorylation on serine/threonine or tyrosine residues or both could be determined with the use of phospho-specific antibodies. Mutatgenesis of potential phosphorylation sites could also be employed to determine which sites are phosphorylated.

In summary, the data in this chapter show that E-cad/CTF2 is ubiquitinated and phosphorylated and both these post-translational modifications impact on the regulation of its nuclear translocation. Cellular signalling pathways are subject to several layers of regulation in order to finely tune the cellular response. Therefore it maybe crucial for the nuclear localisation of E-cad/CTF2 to be tightly controlled by multiple cellular regulators, including p120, RFP, CK1 and other unidentified kinases.

CHAPTER 6

6 NUCLEAR FUNCTIONS OF E-CAD/CTF2

6.1 Introduction

Chapters 4 and 5 describe the regulation of the nuclear localisation of E-cad/CTF2. To understand the significance of E-cad/CTF2 in the nucleus, several studies were carried out to determine its nuclear function. Firstly, an *in vitro* DNA-binding assay was used to show that E-cad/CTF2 can form a complex with DNA. Secondly, transactivation assays were carried out to determine whether E-cad/CTF2 also has transactivation potential. Finally, reporter assays were used to show that E-cad/CTF2 can regulate β-catenin-TCF/LEF-1- and p120-kaiso-mediated signalling pathways in the nucleus.

6.2 *In vitro* DNA binding of E-cad/CTF2

To investigate the role of E-cad/CTF2 in the nucleus, an *in vitro* DNA-binding assay was used to examine whether it can associate with DNA. This assay uses native calf-thymus DNA immobilized on cellulose to precipitate DNA-binding proteins from cell lysates (Hosking et al., 2007; Johnson et al., 2004; Ritt et al., 1998; Surmacz et al., 2002). It is particularly useful when, as in the case of E-cad/CTF2, the potential DNA binding sites are not identified.

6.2.1 E-Cad/CTF2 associates with DNA

The DNA binding activity of E-cad/CTF2 expressed in HEK293 cells was examined. Interestingly, E-cad/CTF2 in the cell lysate bound to DNA-cellulose but not to cellulose alone whereas only very minimal binding of E-cad/CTF3 was detected (Figure 6.1A). This suggests that the N-terminal region of E-cad/CTF2 may be important for this association with DNA since this region is not included in E-cad/CTF3 (see Figure 4.1 for details of E-cad/CTF2 and E-cad/CTF3 constructs). Only the upper, more phosphorylated band of E-cad/CTF2 was precipitated by DNA-cellulose (Figure 6.1A), suggesting that phosphorylation of E-cad/CTF2 maybe important for its DNA-binding. The intensities of Western blot bands were quantified using densitometry, and the binding of E-cad/CTF2 to

DNA was found to be significantly higher than that of E-cad/CTF3 ($p < 0.004$) (Figure 6.1B).

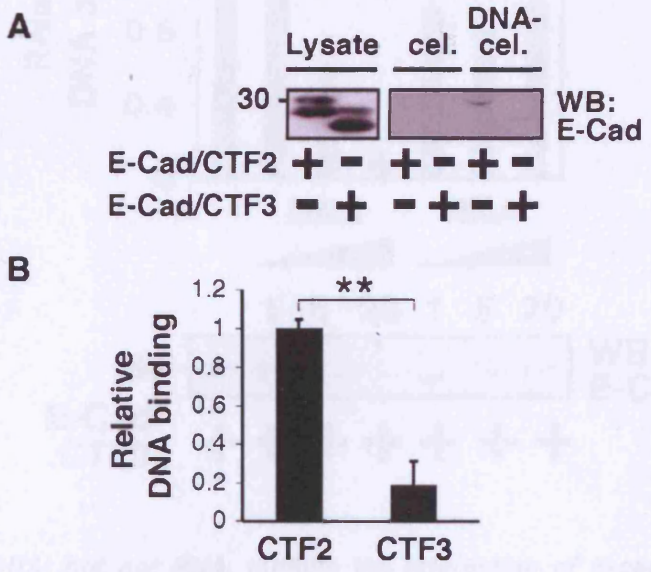


Figure 6.1 ***E-Cad/CTF2 binds to DNA in an in vitro binding assay.*** *(A)and (B) HEK293 cells were transfected with E-cad/CTF2 and E-cad/CTF3 and cell lysates were incubated with cellulose or DNA-cellulose beads. The amount of proteins bound to the beads was examined by Western blotting with anti-E-cadherin antibody. (B) Quantification of E-cad/CTF2 DNA binding. The intensities of Western blot bands were quantified with densitometry from three independent experiments. Error bars indicate SEM. **, $p < 0.004$.*

6.2.2 E-Cad/CTF2 interacts specifically with DNA

The specificity of the interaction between E-cad/CTF2 and DNA was confirmed by performing a competition assay. In this assay, cell lysates were preincubated with increasing amounts of DNA or RNA. Preincubation with DNA suppressed the binding of E-cad/CTF2 to the DNA-cellulose, whereas preincubation with RNA did not (Figure 6.2). This indicates a specific interaction between E-cad/CTF2 and DNA, rather than a non-specific ionic interaction.

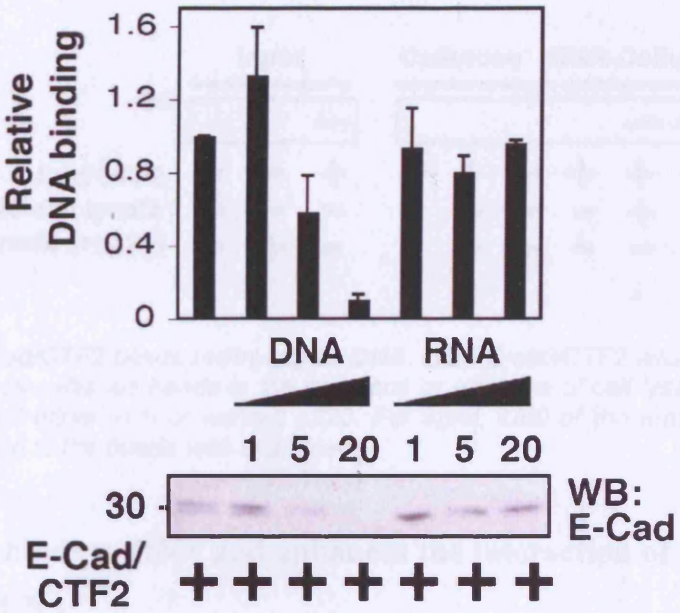


Figure 6.2 ***DNA, but not RNA, inhibits the interaction of E-cad/CTF2 with DNA-cellulose beads****. Lysates of HEK293 cells expressing E-cad/CTF2 were preincubated with 1, 5, or 20 μg of DNA or RNA, followed by incubation with DNA-cellulose beads. The amount of E-cad/CTF2 bound to the beads was examined by Western blotting with anti-E-cadherin antibody. The intensities of Western blot bands were quantified with densitometry from three independent experiments. Quantitation was done jointly with Anthony Wadlow. Error bars indicate SEM.*

6.2.3 E-cad/CTF2 binds indirectly to DNA

Since there are no known DNA binding sites within the E-cadherin cytoplasmic domain, it was examined whether DNA binding was direct by using purified GST-tagged E-cad/CTF2 protein. GST-E-cad/CTF2 alone did not bind to DNA and required HEK293 cell lysate to interact with DNA (Figure 6.3). Lysates from cells overexpressing exogenous p120 further increased the amount of GST-E-cad/CTF2 binding to DNA, suggesting that p120 promotes the interaction between E-cad/CTF2 and DNA (Figure 6.3).

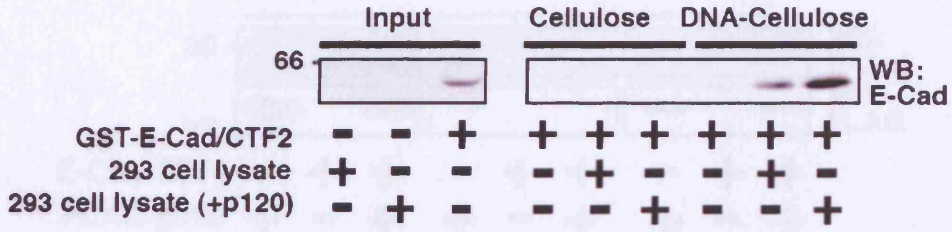


Figure 6.3 ***E-Cad/CTF2 binds indirectly to DNA.*** *GST-E-cad/CTF2 was incubated with cellulose or DNA-cellulose beads in the presence or absence of cell lysates of HEK293 cells transfected either with or without p120. For input, 1/50 of the amount of GST-E-cad/CTF2 added to the beads was examined.*

6.2.4 p120 binds to DNA and enhances the interaction of E-cad/CTF2 with DNA

To further examine the effect of p120 on the DNA binding activity of E-cad/CTF2, E-cad/CTF2 was expressed with or without exogenous p120 in HEK293 cells. Coexpression of p120 substantially increased the association of E-cad/CTF2 with DNA (Figure 6.4 A). Quantification of Western blot bands by densitometry showed that this increase in DNA binding induced by p120 was statistically significant ($p < 0.03$) (Figure 6.4 B). p120 also bound to DNA in this assay (Figure 6.4 A), suggesting that the association of E-cad/CTF2 with DNA could occur via p120.

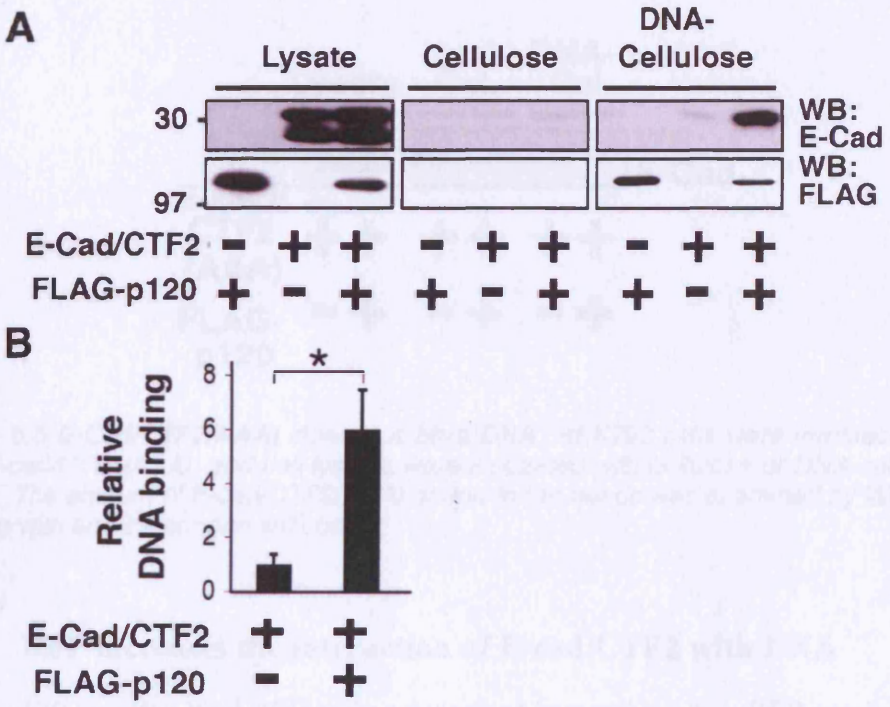


Figure 6.4 ***p120 enhances the interaction of E-cad/CTF2 with DNA.*** *(A) HEK293 cells were transfected with E-cad/CTF2 with or without FLAG-p120, and cell lysates were incubated with cellulose or DNA-cellulose beads. The amount of proteins bound to the beads was examined by Western blotting with the indicated antibodies. Images shown for cellulose and DNA cellulose are from the same film. (B) The intensities of Western blot bands were quantified by densitometry from three independent experiments. Error bars indicate SEM. *, $p < 0.03$*

6.2.5 Direct binding of E-cad/CTF2 to p120 is required for E-cad/CTF2 to associate with DNA

Since p120 enhances the interaction between E-cad/CTF2 and DNA and it is known that E-cad/CTF2 and p120 can bind to each other in the nucleus (Figure 4.15, Chapter 4), it was next examined whether p120 is required for E-cad/CTF2 to bind to DNA. E-cad/CTF2(AAA), which is unable to bind p120, was not detected on the DNA cellulose, even in the presence of exogenous p120 (Figure 6.5). Together with the data in Figure 6.3 and 6.4, this data suggest that E-cad/CTF2 binds to DNA via its interaction with p120.

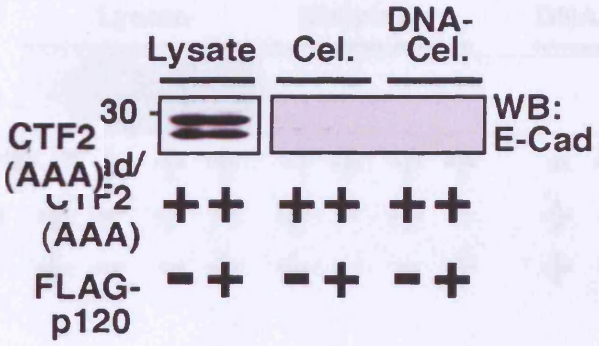


Figure 6.5 ***E-Cad/CTF2(AAA) does not bind DNA.*** *HEK293 cells were transfected as with E-cad/CTF2(AAA), and cell lysates were incubated with cellulose or DNA-cellulose beads. The amount of E-cad/CTF2(AAA) bound to the beads was examined by Western blotting with anti-E-cadherin antibody.*

6.2.6 RFP increases the interaction of E-cad/CTF2 with DNA

Since p120 can also bind RFP and as described in section 1.6.3, RFP can localise in the nucleus and bind DNA, the effect of RFP on the DNA binding activity of E-cad/CTF2 was examined. Coexpression of RFP lead to an increase in the binding of E-cad/CTF2 to DNA, comparable to that observed by coexpression of p120 (Figure 6.6). This suggests that RFP may also be involved in the the DNA binding of the E-cad/CTF2-p120 complex.

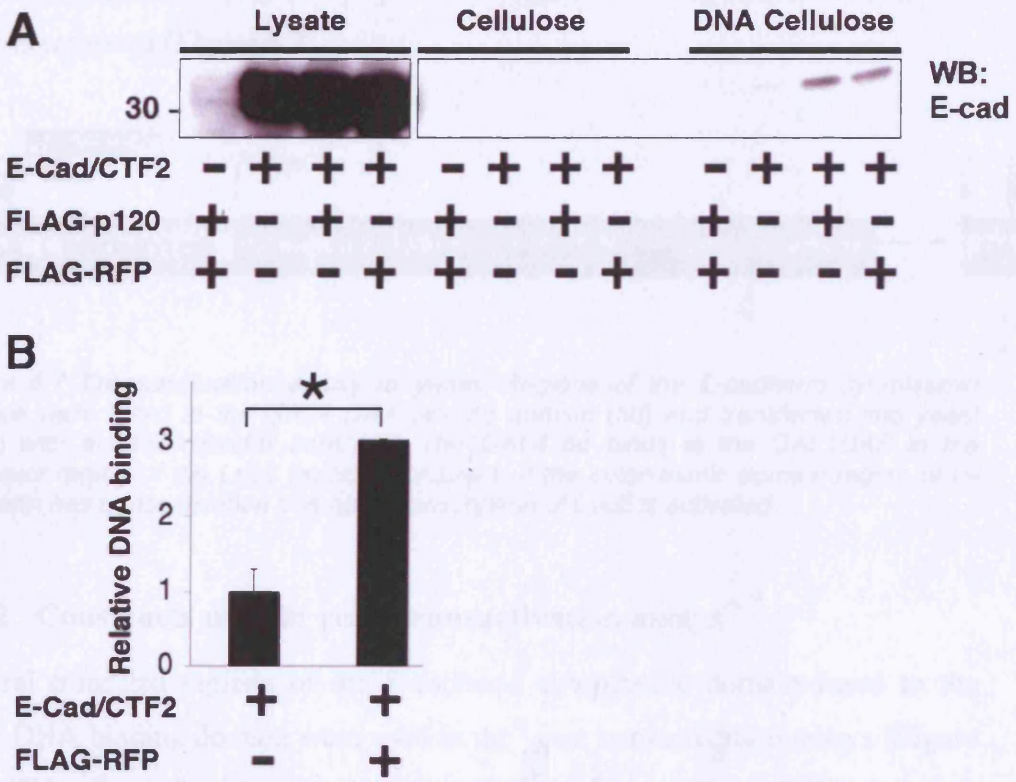


Figure 6.6 ***RFP enhances the interaction of E-cad/CTF2 with DNA.*** *(A) HEK293 cells were transfected with E-cad/CTF2 with or without FLAG-p120 or FLAG-RFP, and cell lysates were incubated with cellulose or DNA-cellulose beads. The amount of E-cad/CTF2 bound to the beads was examined by Western blotting with anti-E-cadherin antibody. (B) The intensities of Western blot bands were quantified by densitometry from two independent experiments. Error bars indicate SEM. *, p < 0.025*

6.3 Analysis of the transactivation potential of E-cad/CTF2

Since E-cad/CTF2 has been shown to bind DNA, it was next examined whether it has transactivating properties. Transactivation assays were carried out in both yeast and in mammalian cells in culture.

6.3.1 Transactivation assay in yeast

The transactivation potential of E-cad/CTF2 was first investigated in yeast. This assay is a variation of the yeast-2-hybrid assay described in Figure 5.8 (chapter 5). Briefly, cytoplasmic domain regions of E-cadherin were fused to the DNA-binding domain (bd) of GAL4 and expressed in yeast, along with a LacZ reporter construct. The GAL4 bd binds to the GAL1UAS in the promotor region of the LacZ reporter construct. If the cytoplasmic region has the ability to recruit

transcriptional machinery to the promotor region, transcription from the *LacZ* gene is activated (Figure 6.7).

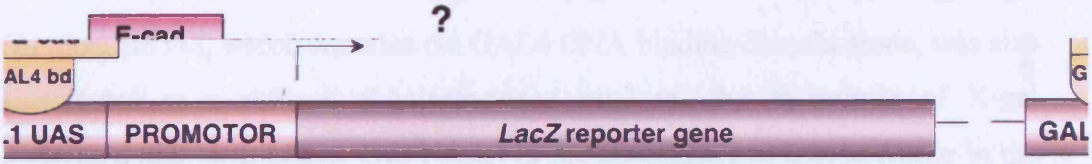


Figure 6.7 ***Transactivation assay in yeast.*** *Regions of the E-cadherin cytoplasmic domain were fused to the GAL4 DNA binding domain (bd) and transfected into yeast along with a LacZ reporter construct. The GAL4 bd binds to the GAL1UAS in the promotor region of the LacZ reporter construct. If the cytoplasmic domain region of E-cadherin has transactivation potential, transcription of LacZ is activated.*

6.3.2 Constructs used in yeast transactivation assays

Several truncated regions of the E-cadherin cytoplasmic domain fused to the Gal4 DNA binding domain were used in the yeast transactivation assays (Figure 6.8). This allows the identification of specific regions of E-cad/CTF2 that may have transactivating properties.

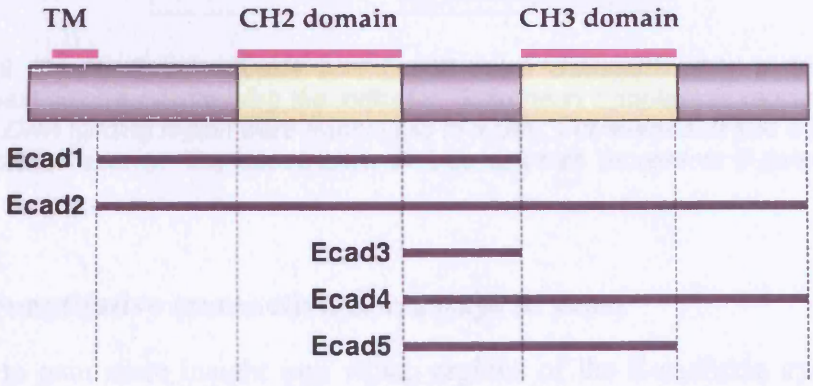


Figure 6.8 ***Regions of E-cadherin fused to the GAL4 DNA binding domain used in yeast transactivation assays.***

6.3.3 Filter assay for β-galactosidase activity in yeast

Transactivation potential of each of the E-cadherin cytoplasmic domain constructs described in Figure 6.8 was first examined using a filter assay for the activity of the *LacZ* gene product β-galactosidase. In this assay yeast colonies

expressing the one of the E-cadherin cytoplasmic domain regions (Ecad1-5) fused to the GAL4 DNA binding domain were transferred to a filter. The cells were then permeabilised and assayed for β-galactosidase activity using X-gal solution. pBTM, which contains the GAL4 DNA binding domain alone, was also transfected as a control. β-galactosidase catalyses the hydrolysis of X-gal yielding a blue precipitate. This results in the appearance of a blue colour in the colonies. Using this technique it was shown that all the E-cadherin cytoplasmic domain regions, except for Ecad3, have transactivating potential in yeast (Figure 6.9). However, this technique is qualitative and does not provide any quantitative information about the amount of transcriptional activation.

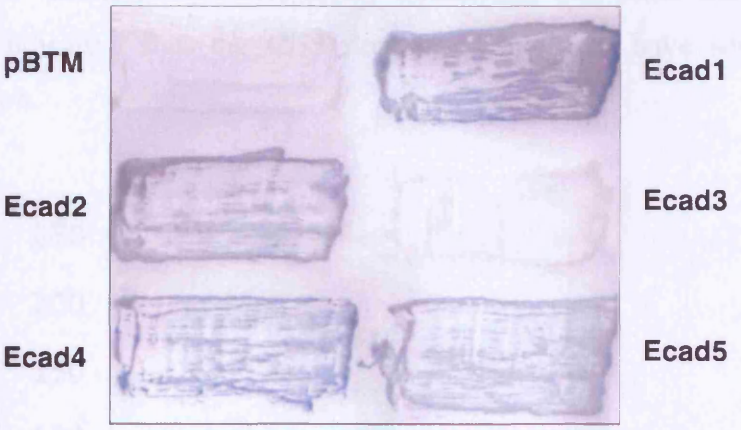


Figure 6.9 ***Ecad1, Ecad2, Ecad4 and Ecad5 have transactivating properties in yeast.*** *Yeast colonies expressing the indicated E-cadherin cytoplasmic region fused to the GAL4 DNA binding region were transferred to a filter, permeabilised and assayed for β-galactosidase activity. The appearance of blue colonies represents β-galactosidase activity.*

6.3.4 Quantitative transactiviation assays in yeast

In order to gain more insight into which regions of the E-cadherin cyoplasmic domain are responsible for the transactivating property in yeast, a quantitative liquid culture assay for β-galactosidase assay was performed. In this assay the release of o-nitrophenol from the β-galactosidase substrate o-nitrophenylgalactosidase (ONPG) is measured. For more details see section 2.4.5 (Materials and Methods). Liquid cultures of yeast expressing the E-cadherin cytoplasmic domain constructs described in Figure 6.8 fused to the GAL4 DNA binding domain. pBTM was also transfected as a negative control. The results of this assay showed Ecad2 and Ecad4 had the highest transactivating property

(Figure 6.10). Ecad4 lacks the N-terminal region including the CH2 domain. This suggests that the CH2 domain and the N-terminal region are not required for transactivation. As expected from the qualitative filter assay Ecad3 has no transactivation activity (Figure 6.10). Interestingly, Ecad1, in which the region of E-cadherin from the beginning of the CH3 domain to the C-terminal is deleted, shows very weak transactivation (Figure 6.10). This suggests that the C-terminal end and the CH3 domain may be responsible for most of the transactivation property of the E-cadherin intracellular domain. Ecad5, which differs only from Ecad4 by the loss of the very C-terminal region but not the CH3 domain, showed a large reduction in transactivation (Figure 6.10). This suggests that this region is important for transactivation. However, Ecad5 still has some transactivation properties, indicating that the CH3 domain is likely to have some role in transactivation.

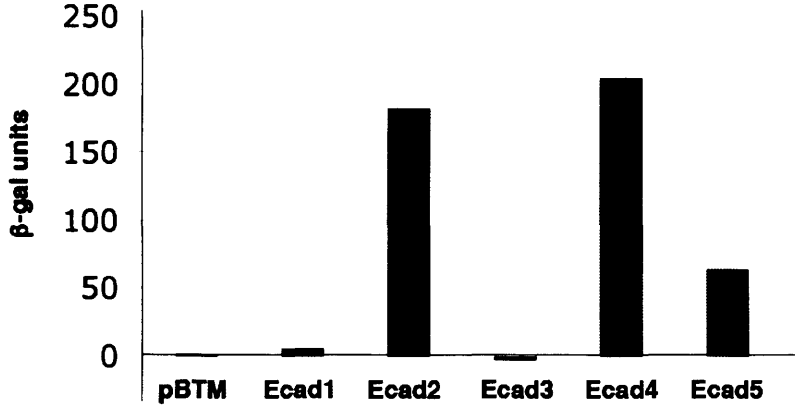


Figure 6.10 ***Quantification of the transactivating properties of regions of the E-cadherin cytoplasmic domain in yeast.*** *Liquid cultures of yeast expressing the indicated E-cadherin cytoplasmic region fused to the GAL4 DNA binding region were assayed for β-galactosidise activity. β-galactosidase activity is expressed in β-gal units (see section 2.4.5, Materials and Methods, for more details).*

6.3.5 Mammalian transactivation assay

In order to determine whether the transactivating properties of the E-cadherin intracellular domain are also observed in mammalian cells, a transactivation assay was performed in HEK293 cells. Ecad2 (described in Figure 6.8) was fused to the GAL4 DNA binding domain in a mammalian expression vector (see

table 2.7, section 2.1.11, Materials and Methods for details). This region was chosen as it is equivalent to the region of E-cadherin found in the fragment E-cad/CTF2. The principle of the mammalian transactivation assay is very similar to the yeast transactivation assay and is shown in Figure 6.11. Briefly, HEK293 cells were transfected with E-cad/CTF2 fused to the GAL4 DNA binding domain, along with a reporter construct containing the firefly *luciferase* gene and the GAL4 UAS in the promotor region. The GAL4 DNA binding domain binds to the GAL4 UAS and if E-cad/CTF2 has transactivating properties, *luciferase* is transcribed.

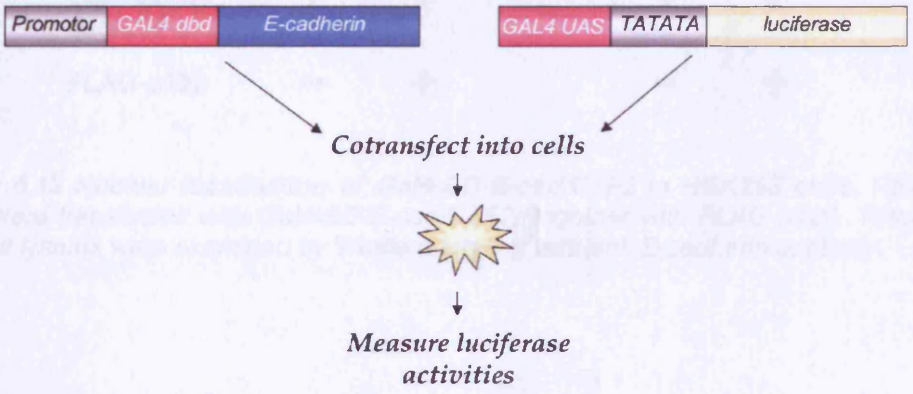


Figure 6.11 ***Transactivation assay in mammalian cells.***

6.3.6 Transactivation was not induced by E-cad/CTF2 in mammalian cells

A transactivation assay was carried out as depicted in Figure 6.11 using E-cad/CTF2 fused to the DNA binding domain of GAL4 in HEK293 cells. Firstly it was confirmed that E-cad/CTF2 localises to the nucleus when fused to the GAL4 DNA binding domain. A substantial amount of E-cad/CTF2-GAL4dbd was detected in the nuclear fraction of HEK293 cells even in the absence of exogenous p120 (Figure 6.12). This is likely to be because the DNA binding domain of GAL4 contains an NLS sequence. Transactivation was not detected when E-cad/CTF2 fused to GAL4dbd was expressed in HEK293 cells, compared to the control plasmid containing the GAL4 DNA binding domain alone (Figure 6.13). Interestingly E-cad/CTF2 showed a decrease in reporter activity compared to the control (Figure 6.13). This may indicate that in mammalian cells, E-

cad/CTF2 has a repressive activity, in contrast to its transactivating property in yeast cells. However it is difficult to firmly conclude this from this experiment alone as any transcription in the control conditions would be at very low basal levels. It may be that E-cad/CTF2 has different binding partners in yeast and mammalian cells which could explain the differences in the results of transactivation assays between the two systems.

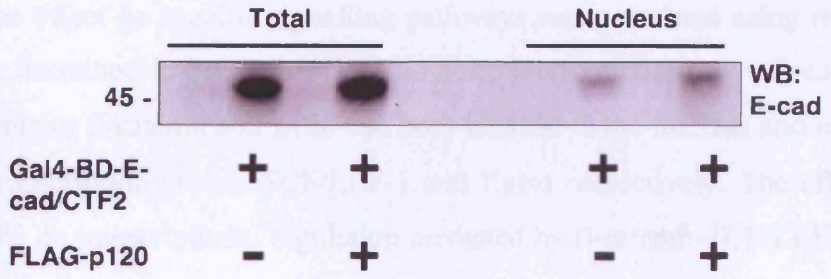


Figure 6.12 ***Nuclear localisation of Gal4-BD-E-cad/CTF2 in HEK293 cells.*** *HEK293 cells were transfected with Gal4-BD-E-cad/CTF2, together with FLAG-p120. Total and nuclear lysates were examined by Western blotting with anti E-cadherin antibody.*

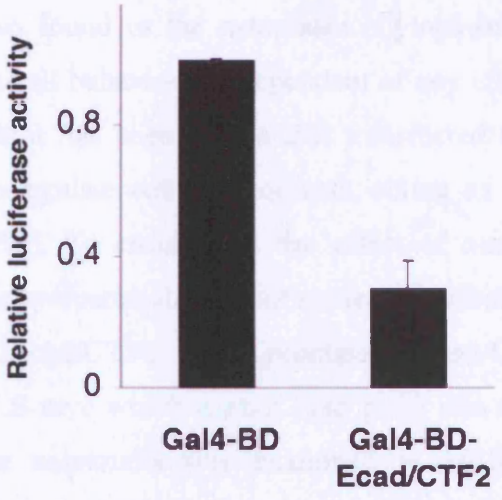


Figure 6.13 ***Transactivation was not induced by E-cad/CTF2 in HEK293 cells.*** *HEK293 cells were transfected with either Gal4-BD alone or Gal4-BD-E-cad/CTF2, together with the luciferase reporter construct and renilla luciferase, and luciferase activites were measured. Relative luciferase activity indicates the ratio of firefly/renilla luciferase activities from three independent experiments. Error bars indicate standard deviation.*

6.4 Effect of E-cad/CTF2 on transcription from β-catenin/TCF/LEF-1- and p120/Kaiso-regulated promoters

Since the mammalian transactivation assays did not provide conclusive insight into the possible role of E-cad/CTF2 in the regulation of transcription in the nucleus, the effect on specific signalling pathways was examined using reporter assays. As described in Sections 1.3.4 and 1.3.5 (Introduction), two E-cadherin binding proteins β-catenin and p120 can both localise to the nucleus and interact with the transcription factors TCF/LEF-1 and Kaiso respectively. The effect of E-cad/CTF2 on transcriptional regulation mediated by β-catenin-TCF/LEF-1 and p120-Kaiso was examined.

6.4.1 Production of nuclear-localised E-cad/CTF2

As shown in Figures 4.3 - 4.6 (Chapter 4), even when E-cad/CTF2 is localised in the nucleus, it is also found in the cytoplasm. Cytoplasmic E-cad/CTF2 may have other effects on cell behaviour, independent of any effects that it has in the nucleus. For example it has been shown that transfected cytoplasmic cadherin sequences can down-regulate cell-cell contacts, acting as a dominant negative (Nieman et al., 1999a). To ensure that the effect of nuclear E-cad/CTF2 on transcription could be evaluated alone, four nuclear localisation sequences (NLS) were fused to E-cad/CTF2, to produce E-cad/CTF2-NLS-myc. E-Cad/CTF2(AAA)-NLS-myc which cannot bind p120 was also constructed. The localisation of these constructs was examined in HEK293 cells. Both E-cad/CTF2-NLS and E-cad/CTF2(AAA)-NLS were found to localise in the nucleus and cytoplasmic staining was not detected (Figure 6.14).

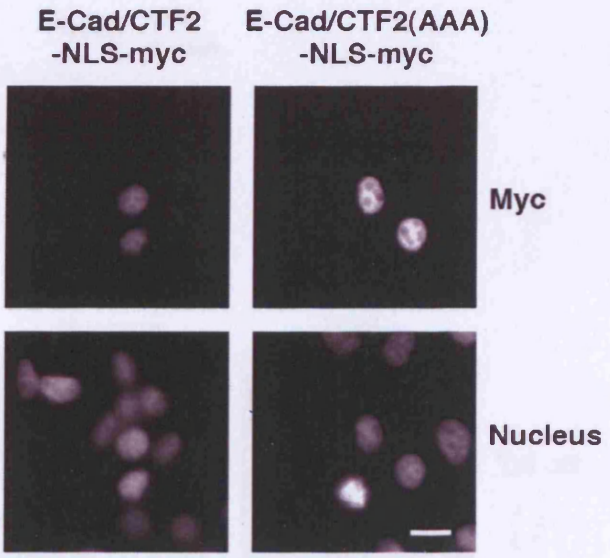


Figure 6.14 ***Nuclear localisation of E-cad/CTF2-NLS-myc and E-cad/CTF2(AAA)-NLS-myc.*** *HEK293 cells were transfected with E-cad/CTF2-NLS-myc and E-cad/CTF2(AAA)-NLS-myc. Immunostaining was performed with anti-myc antibody. Nuclei were stained with Hoechst dye. Images were acquired using epifluorescent microscopy. Immunostaining was done jointly with Anthony Wadlow. Scale bar represents 10 μm.*

In order to reveal other functions of E-cad/CTF2 in the nucleus, MDCK cell lines stably expressing GFP-E-cad/CTF2-NLS in a tetracycline-inducible manner were established. These cells express GFP-E-cad/CTF2-NLS predominantly in the nucleus upon treatment with tetracycline (Figure 6.15 A and B). No clear differences in cellular phenotype could be observed by analysis with light microscopy, but a more detailed assessment of their phenotype is required. These cells could be a useful tool for the identification of novel targets of nuclear E-cad/CTF2.

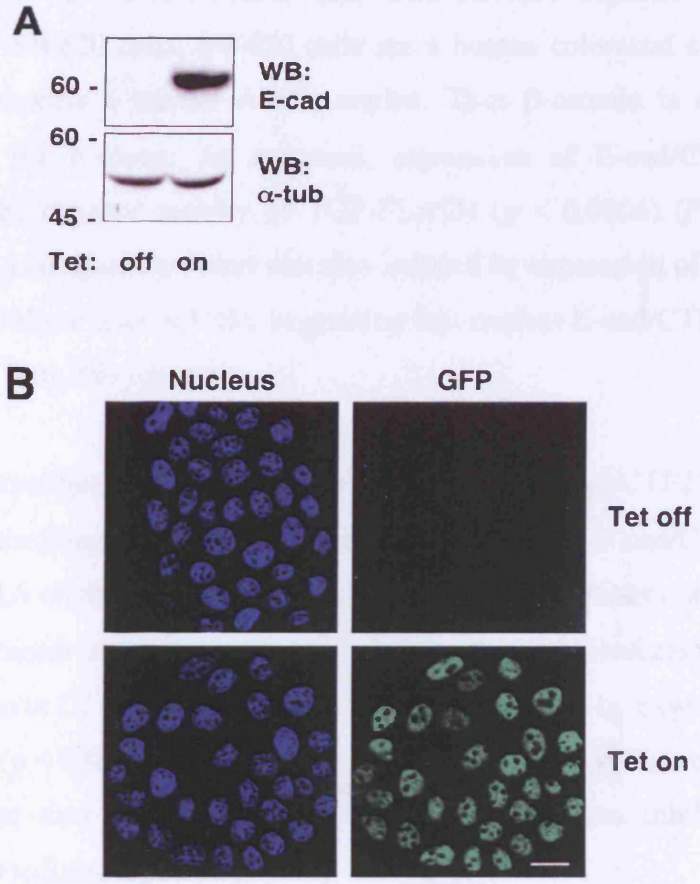


Figure 6.15 ***Characterisation of MDCK cells stably expressing GFP-E-cad/CTF2-NLS in a tetracycline-inducible manner.*** *(A and B) An MDCK cell clone stably expressing tetracycline-inducible GFP-E-cad/CTF2-NLS was treated with tetracycline (2 μg/ml) for 24 hours. (A) Total cell lysates were examined by Western blotting with anti-E-cadherin antibody. (B) Nuclei were stained with Hoechst dye. Images were acquired using confocal microscopy. Scale bar represents 15 μm.*

6.4.2 E-Cad/CTF2 in the nucleus represses transcription from β-catenin-TCF-regulated promoters

To examine β-catenin-mediated Wnt signalling pathways, a TCF/LEF-1 reporter plasmid TOP-FLASH was used. This contains TCF/LEF-1 binding sites upstream of the *luciferase* reporter gene (Figure 6.16A). FOP-FLASH was used as a negative control which contains a mutation in the TCF/LEF-1 binding sites (Figure 6.16A). As described in section 1.3.4 (Introduction), β-catenin binds to TCF/LEF-1 at the promotor region and activates transcription. TCF/LEF-1 and cadherins have been shown to compete for binding to β-catenin (Gottardi et al., 2001; Graham et al., 2000; Sadot et al., 1998). The effect of E-cad/CTF2 and E-

cad/CTF2-NLS on TOP-FLASH and FOP-FLASH reporter activity was examined in SW620 cells. SW620 cells are a human colorectal carcinoma cell line which express a mutant APC complex. Thus β-catenin is stabilized and enriched in the nucleus. As expected, expression of E-cad/CTF2 strongly suppressed the reporter activity of TOP-FLASH ($p < 0.0006$) (Figure 6.16B). Importantly, a comparable effect was also induced by expression of E-cad/CTF2-NLS ($p < 0.006$) (Figure 6.16B), suggesting that nuclear E-cad/CTF2 can inhibit transcription from this reporter.

To further investigate the inhibitory role of nuclear E-cad/CTF2 in β-catenin-TCF/LEF-1-mediated gene expression, the effect of E-cad/CTF2 and E-cad/CTF2-NLS on the expression of β-catenin responsive genes *c-myc* and *cyclin D1* was examined using reporter assays in SW620 cells. Transcription from the *c-myc* and *cyclin D1* promoters was significantly repressed by expression of both E-cad/CTF2 ($p < 0.005$) and E-cad/CTF2-NLS ($p < 0.0005$) (Figure 6.17). Taken together these data suggest that nuclear E-cad/CTF2 can inhibit β-catenin-TCF/LEF-1-mediated signalling.

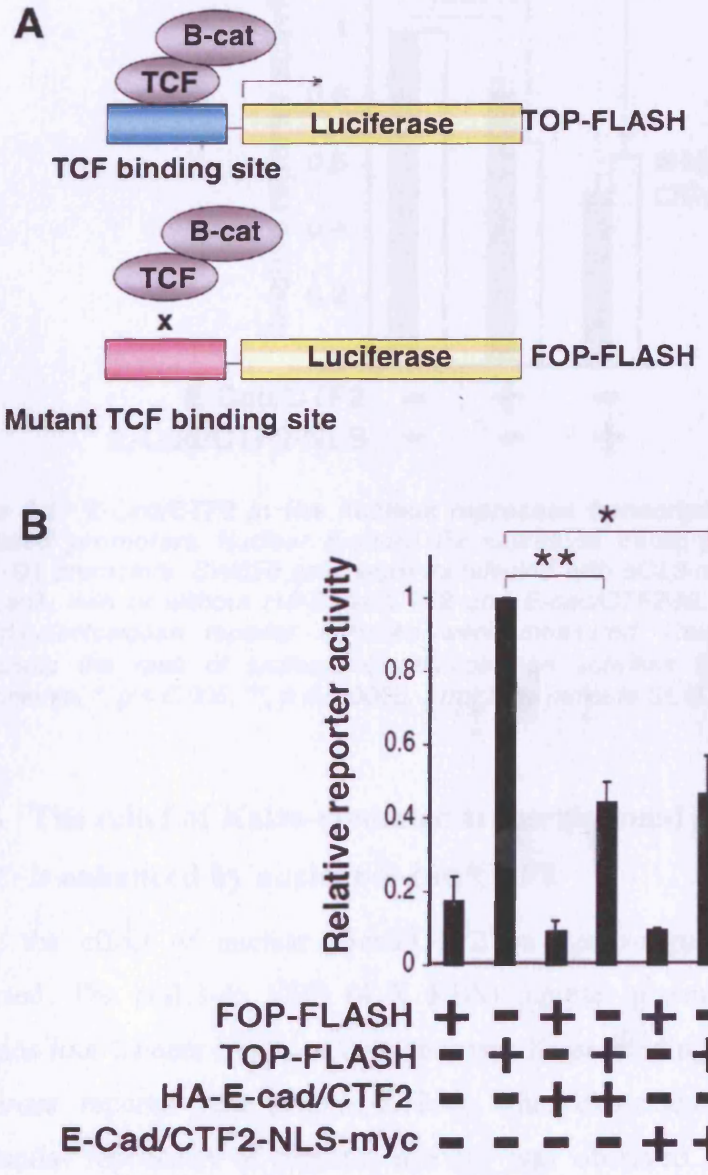


Figure 6.16 ***E-cad/CTF2 in the nucleus represses transcription of β-catenin-TCF/LEF-1 target genes in SW620 cells.*** *(A) TOP-FLASH and FOP-FLASH reporter plasmids. β-catenin binds to TCF/LEF-1 and activates transcription from the TOP-FLASH reporter plasmid. FOP-FLASH contains mutant TCF binding sites in the promotor region. (B) SW620 cells were transfected with pTOP-FLASH, pFOP-FLASH, and LacZ, with or without HA-E-cad/CTF2 and E-cad/CTF2-NLS-myc, and luciferase and β-galactosidase reporter activities were measured. Relative reporter activity represents the ratio of luciferase/β-galactosidase activities from three independent experiments. *, $p < 0.006$; **, $p < 0.0006$. Error bars indicate SEM.*

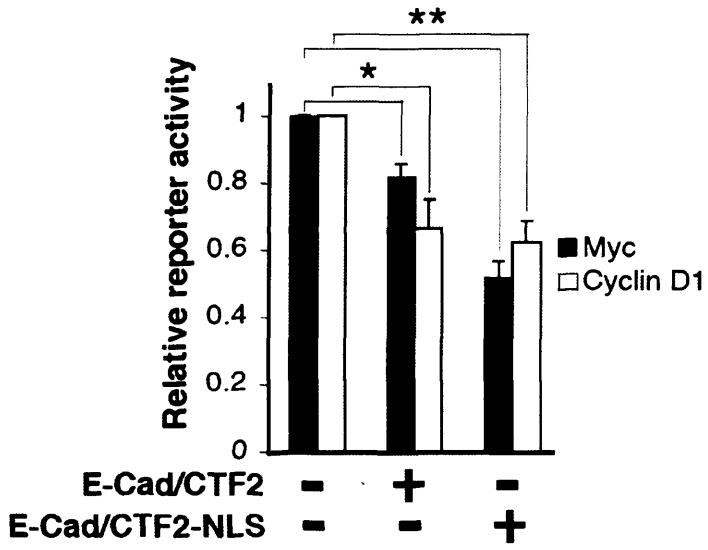


Figure 6.17 ***E-Cad/CTF2 in the nucleus represses transcription from TCF/LEF-1-regulated promoters*** *Nuclear E-cad/CTF2 supresses transcription from c-myc and cyclin D1 promoters. SW620 cells were transfected with pGL3-myc or pGL3-cyclin D1 and LacZ, with or without HA-E-cad/CTF2 and E-cad/CTF2-NLS-myc, and luciferase and β-galactosidase reporter activities were measured. Relative reporter activity represents the ratio of luciferase/β-galactosidase activities from five independent experiments. *, $p < 0.005$; **, $p < 0.0005$. Error bars indicate SEM.*

6.4.3 The relief of Kaiso-mediated transcriptional repression by p120 is enhanced by nuclear E-cad/CTF2

Next, the effect of nuclear E-cad/CTF2 on Kaiso-regulated promoters was analysed. The pGL3-4x KBS (4 X KBS) reporter plasmid was used, which contains four tandem copies of the consensus Kaiso binding site upstream of the *luciferase* reporter gene (Figure 6.18A). When expressed in HEK293 cells, a substantial repression of reporter activity was observed from the 4 X KBS construct, compared to the pGL3 control vector alone (Figure 6.18B), suggesting repression of transcription mediated by endogenous Kaiso protein binding to Kaiso binding sites in the promotor region. With the 4 X KBS construct, the effects of p120, E-cad/CTF2, and E-cad/CTF2-NLS on reporter activity were examined in HEK293 cells. As previously shown (Kelly et al., 2004), a significant increase in reporter activity was observed in the presence of exogenous p120, indicating that p120 relieved the repression mediated by endogenous Kaiso protein ($p < 0.038$) (Figure 6.19). Interestingly, this p120-mediated increase in reporter activity was significantly enhanced by coexpression of E-cad/CTF2 ($p < 0.037$) (Figure 6.19). Enhancement of reporter

activity was also observed with E-cad/CTF2-NLS ($p < 0.025$), suggesting that this effect of E-cad/CTF2 can indeed occur in the nucleus (Figure 6.19). Expression of E-cad/CTF2(AAA)-NLS did not enhance reporter activity (Figure 6.19), suggesting that p120 binding is required for E-cad/CTF2 to enhance transcription.

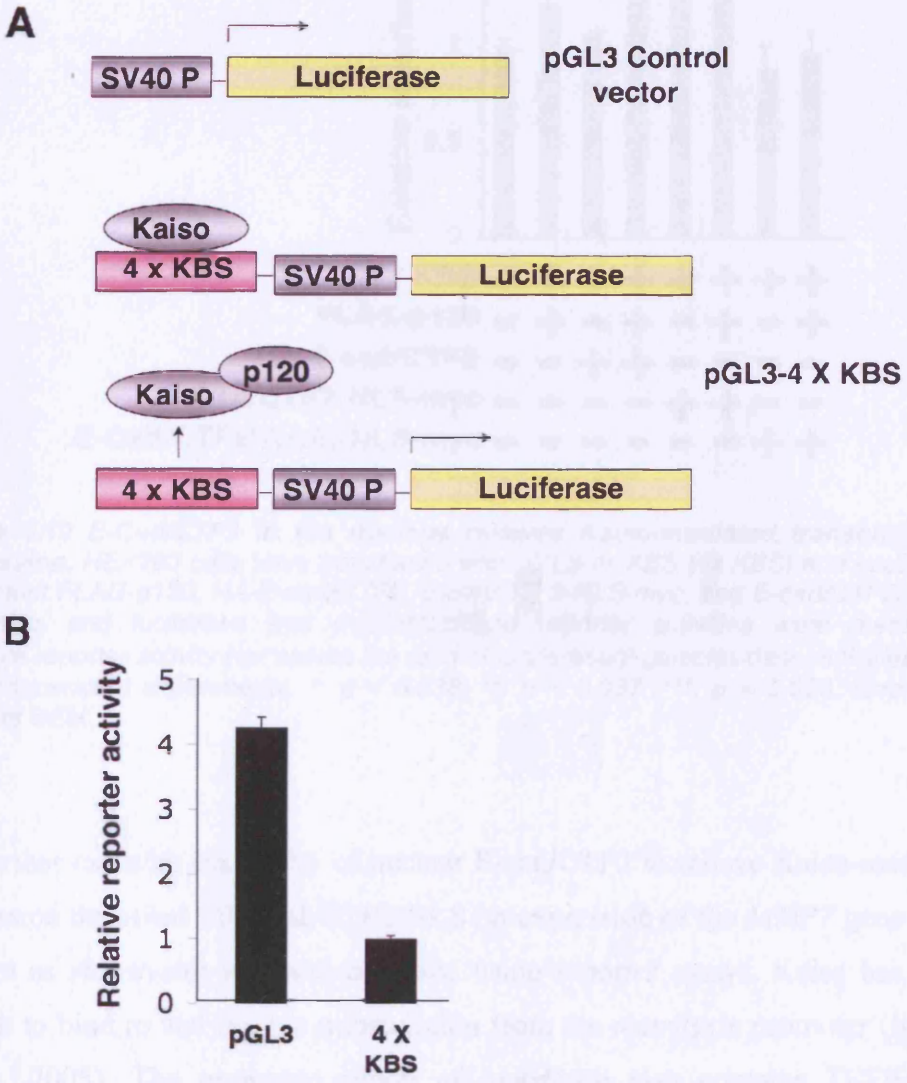


Figure 6.18 ***Reporter assays using pGL3- 4 X KBS*** *(A)The addition of 4 X Kaiso binding sites (KBS) to the promotor region of the pGL3 control luciferase reporter vector results in suppression of transcription due to the recruitment of the transcriptional repressor Kaiso. p120 relieves Kaiso-mediated repression by releasing Kaiso from the DNA (B) HEK293 cells were transfected with pGL3, pGL3- 4 X KBS (4 X KBS) and LacZ, and luciferase and β-galactosidase reporter activities were measured. Relative reporter activity represents the ratio of luciferase/β-galactosidase activities from three independent experiments. Error bars indicate SEM.*

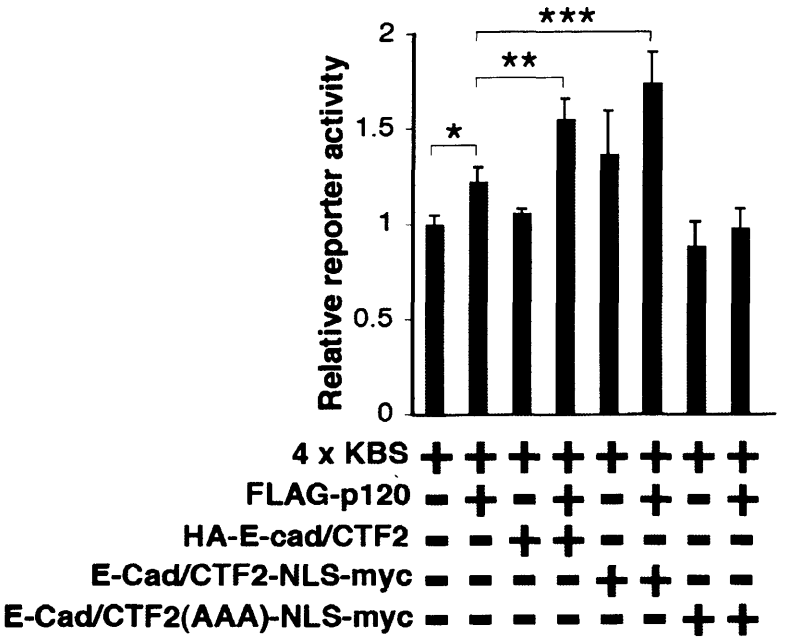


Figure 6.19 ***E-Cad/CTF2 in the nucleus relieves Kaiso-mediated transcriptional repression.*** *HEK293 cells were transfected with pGL3-4x KBS (4x KBS) and LacZ, with or without FLAG-p120, HA-E-cad/CTF2, E-cad/CTF2-NLS-myc, and E-cad/CTF2(AAA)-NLS-myc, and luciferase and β-galactosidase reporter activities were measured. Relative reporter activity represents the ratio of luciferase/β-galactosidase activities from five independent experiments. *, $p < 0.038$; **, $p < 0.037$; ***, $p < 0.025$. Error bars indicate SEM.*

To further examine the ability of nuclear E-cad/CTF2 to relieve Kaiso-mediated repression the effect of E-cad/CTF2-NLS on expression of the *MMP7* gene (also known as *matrilysin*) was also analysed using reporter assays. Kaiso has been shown to bind to and repress transcription from the *matrilysin* promoter (Spring et al., 2005). The promoter region of *matrilysin* also contains TCF/LEF-1 binding sites. To exclude the influence of TCF/LEF-1, HEK293 cells were used in which β-catenin is not localised in the nucleus. Expression of p120 or E-cad/CTF2-NLS alone had no effect, but coexpression of E-cad/CTF2-NLS and p120 significantly enhanced transcription from the *matrilysin* promotor (2.3 kb Hmat) in HEK293 cells ($p < 0.006$) (Figure 6.20). E-Cad/CTF2(AAA)-NLS had no effect on transcription from this promotor (Figure 6.20), suggesting that p120 binding is required for nuclear E-cad/CTF2 to enhance transcription of the *matrilysin* gene.

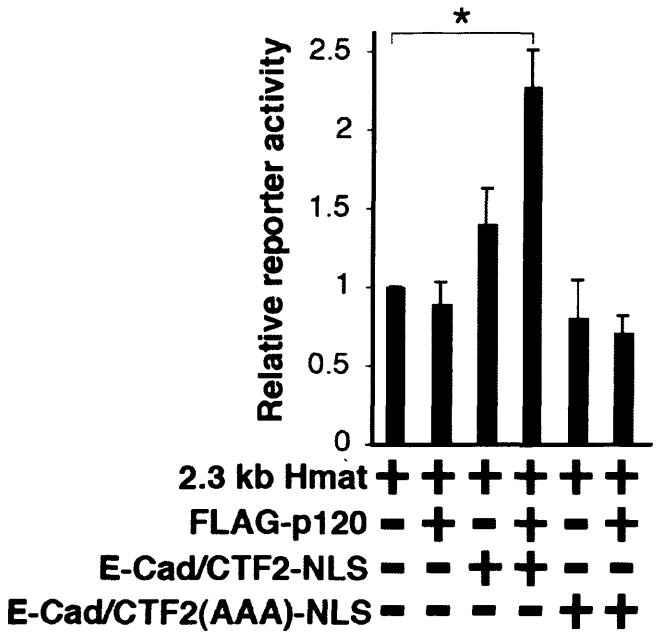


Figure 6.20 ***Nuclear E-cad/CTF2 enhances transcription of matrilysin****. HEK293 cells were transfected with a matrilysin promoter-luciferase reporter containing the 2.3 kb human matrilysin promoter (pGL3-Basic-2.3kb HMat) and LacZ, with or without FLAG-p120, E-cad/CTF2-NLS-myc, and E-cad/CTF2(AAA)-NLS-myc, and luciferase and β-galactosidase reporter activities were measured. Relative reporter activity represents the ratio of luciferase/β-galactosidase activities from three independent experiments. *, $p < 0.006$. Error bars indicate SEM.*

6.4.4 E-Cad/CTF2 interacts with Kaiso in the presence of p120

Since p120 relieves Kaiso-mediated repression through binding to Kaiso, it was examined whether E-cad/CTF2 can also bind to Kaiso, using a GST pull-down assay. Exogenous Kaiso protein expressed in HEK293 cells specifically bound to GST-E-cad/CTF2, only in the presence of exogenous p120 (Figure 6.21). Collectively, the data in sections 6.4.3 and 6.4.4 suggest that E-cad/CTF2 forms a complex with Kaiso via p120 and that E-cad/CTF2 can enhance the effect of p120 on Kaiso-mediated gene regulation.

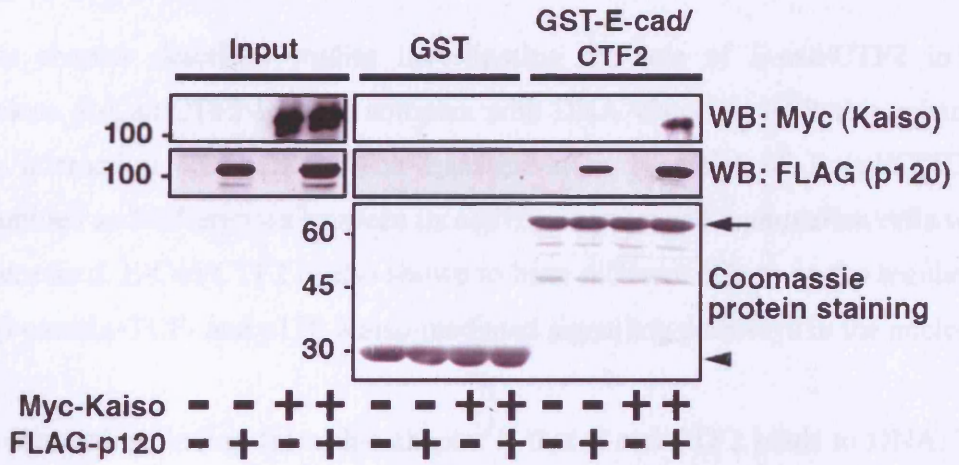


Figure 6.21 ***E-Cad/CTF2 interacts with Kaiso in the presence of p120.*** *Beads coupled to GST or GST-E-cad/CTF2 were incubated with lysates of HEK293 cells expressing myc-Kaiso and/or FLAG-p120. The proteins bound to the beads were analysed by Coomassie staining and Western blotting with anti-myc and anti-FLAG antibodies. The arrow and arrowhead indicate the positions of GST-E-cad/CTF2 and GST, respectively. By kind permission of Dr. Mihoko Kajita.*

6.5 Discussion

This chapter describes studies investigating the role of E-cad/CTF2 in the nucleus. E-Cad/CTF2 forms a complex with DNA via p120. RFP also enhances the interaction with DNA. The transactivation potential of E-cad/CTF2 is examined and differences between its activity in yeast and mammalian cells were determined. E-Cad/CTF2 is also shown to have different effects on the regulation of β-catenin-TCF- and p120-Kaiso-mediated signalling pathways in the nucleus.

An interesting finding from this chapter is that E-cad/CTF2 binds to DNA. The association of E-cad/CTF2 and DNA is not direct, as E-cad/CTF2 requires p120 for its DNA binding. It is not clear whether p120 binds directly to DNA. This could be addressed with the use of purified p120 protein. Only the more phosphorylated form of E-cad/CTF2 was detected in the complex with DNA, indicating that phosphorylation could also be involved in the regulation of the DNA-binding activity of E-cad/CTF2. RFP expression also enhances the DNA binding of E-cad/CTF2. It is important to determine whether RFP is also found in the complex with DNA and whether E-cad/CTF2, p120 and RFP can form a trimeric complex. As described in section 1.6.3 (Introduction), RFP is found in the nucleus and has been shown to have both transcriptional repressive and activating activities, as well as being present at sites of transcriptional regulation. Knocking down the expression of RFP with RNAi would provide some insight into whether RFP is required for the DNA-binding of E-cad/CTF2. It is also not clear whether the enhancement of the DNA-binding of E-cad/CTF2 by RFP involves ubiquitination. Investigation of the effect of ubiquitin and mutations within the RING finger domain of RFP on the DNA binding of E-cad/CTF2 could clarify on this issue. At present, the DNA binding site of the E-cad/CTF2-p120 complex is not clear. It is unlikely that it binds to the Kaiso-binding regions because p120 promotes dissociation of Kaiso from DNA (Daniel et al., 2002). Thus, the E-cad/CTF2-p120 complex may be involved in the regulation of transcription or chromatin remodeling of unknown gene(s). Determination of DNA binding sites could be addressed by performing CASTing (cyclic amplification and selection of targets) experiments or by ChIP cloning.

It was found that the region of E-cadherin equivalent to E-cad/CTF2 has a transactivating property in yeast. This seemed particularly interesting as the regions of E-cad/CTF2 that are important for transactivation and DNA-binding are at opposite ends of the protein fragment. The CH3 domain and the most C-terminal region were most important for transactivation. DNA binding seems to be mediated through the CH2 domain or the most N-terminal region of E-cad/CTF2, since E-cad/CTF3 showed very weak DNA binding activities. However, transactivation by E-cad/CTF2 was not observed in mammalian cells. In fact, a decrease in transcription of the reporter compared to the control was observed in transactivation assays in HEK293 cells. Preliminary studies using a form of E-cad/CTF2 that does not contain the CH3 domain and the most C-terminal region, showed that this truncated form of E-cad/CTF2 showed less repression. The apparent discrepancies between the results of the transactivation assays in yeast and mammalian cells could be due to different binding proteins of the cytoplasmic domain of E-cadherin and differences in the transcriptional machinery. However it is difficult to conclude that E-cad/CTF2 has a general repressive activity in mammalian cells as the level of transcription from the control reporter plasmid containing only the Gal4 bd is only at very low basal levels. It is more useful to analyse potential effects of E-cad/CTF2 on transcription by the identification of specific target genes.

As a first step towards identifying E-cad/CTF2-mediated nuclear signalling pathways, the effect of E-cad/CTF2 on transcriptional regulation by two of its nuclear binding partners, β-catenin and p120, was analysed. E-Cad/CTF2 was shown to suppress β-catenin-mediated signalling pathways with reporter assays using the TOP-FLASH reporter, and with cyclin D1 and myc promoter reporters in SW620 cells. With NLS-tagged E-cad/CTF2, it was demonstrated that this suppression can occur in the nucleus. This is expected, since TCF/LEF-1 and E-cadherin compete for binding to β-catenin (Gottardi et al., 2001; Graham et al., 2000; Sadot et al., 1998). Recently, it has been shown that the γ-secretase-mediated cleavage product of N-cadherin, N-cad/CTF2, is also localised in the nucleus and regulates β-catenin-mediated signalling (Shoval et al., 2007; Uemura et al., 2006a). However, as discussed in section 4.6, there are functional

differences between E-cad/CTF2 and N-cad/CTF2. In addition to different cellular localisations, N-cad/CTF2 enhances canonical Wnt-signalling pathways (Shoval et al., 2007; Uemura et al., 2006a), while E-cad/CTF2 suppresses it. As suggested in section 4.6 (Chapter 4), E-cad/CTF2 and N-cad/CTF2 may have different specific binding proteins, which may mediate differences in their effect on transcription as well as nuclear translocation.

With reporter assays, using the 4 X KBS reporter and the matrilysin promoter reporter in HEK293 cells, it is also shown that the expression of E-cad/CTF2 potentiates the effect of p120 on the relief of Kaiso-mediated transcriptional repression. With GST pull-down assays, it was shown that E-cad/CTF2 forms a trimeric complex with p120 and Kaiso. These data suggest that the interaction between these three proteins influences gene transcription. It would be interesting to determine whether E-cad/CTF2 can strengthen the binding of p120 with Kaiso thereby enhancing its ability to dissociate Kaiso from DNA. This could be addressed with ChIP assays.

To determine whether β-catenin-TCF/LEF-1 and p120-Kaiso gene targets are transcriptionally regulated by E-cad/CTF2, the levels of endogenous gene targets by reverse-transcriptase (RT)-PCR should be analysed. Many of the targets of canonical Wnt signalling are also regulated by Kaiso. Since E-cad/CTF2 appears to have opposite effects on TCF/LEF-1 and Kaiso, it may provide the cell with one way to separate the activities of these pathways. As described in sections 1.3.4 and 1.3.5 (Introduction), there are differences in the activation of β-catenin-TCF/LEF-1- and p120-Kaiso-mediated signalling. In particular, while p120 and β-catenin are both released into the cytosol in conditions where E-cadherin is down-regulated, β-catenin is rapidly degraded and requires cellular activation with Wnt for its stabilization. p120 is more stable in the cytosol, although a recent study has shown that Wnt signals can lead to p120 stabilisation and enhancement of Kaiso sequestration from DNA (Park et al., 2006). In the future, other downstream targets of E-cad/CTF2 could be identified by using the MDCK cell line established in this study which stably expresses E-cad/CTF2-NLS under

a tetracycline-inducible promoter. These cells could be used in a genomic or proteomic array to identify novel targets.

CHAPTER 7

7 FINAL DISCUSSION

In this thesis the role of the γ-secretase cleavage product of E-cadherin (E-cad/CTF2) in signalling is examined. It is shown that stimuli inducing an EMT in MDCK cells, treatment with HGF or activation of Ras, lead to the production of E-cad/CTF2. The crucial role of p120 in the nuclear localisation of E-cad/CTF2 is demonstrated using several experimental systems. Both phosphorylation and ubiquitination (possibly mediated by a novel ubiquitin E3 ligase RFP) are shown to be involved in the regulation of the nuclear localisation of E-cad/CTF2. Furthermore, E-cad/CTF2 forms a complex with DNA via p120. DNA binding of E-cad/CTF2 is also enhanced by RFP. Moreover nuclear E-cad/CTF2 inhibits the activation of TCF/LEF-1-regulated promoters by β-catenin. In addition it is also shown that E-cad/CTF2 acts in concert with p120 to relieve transcriptional repression mediated by Kaiso.

As a signalling molecule, E-cadherin has the potential to provide information to the cell about the status of cell-cell contacts. Cleavage of E-cadherin at the membrane results in production of E-cad/CTF2 which can localise in the nucleus and modulate gene transcription. It will be important to determine whether this pathway plays a role in relaying signals from the membrane to the nucleus about the level of adhesion between cells and their neighbours.

As E-cad/CTF2 is released under conditions inducing an EMT, it is tempting to speculate that E-cad/CTF2's role in signalling to the nucleus could be involved in this morphological transition. It is known that γ-secretase-mediated cleavages are strongly enhanced by upregulation of MMPs or ADAMs which remove the ectodomain of γ-secretase's transmembrane protein substrate (Struhl and Adachi, 2000). MMPs are involved in EMT and also in the progression to metastatic cancers (Lee and Murphy, 2004; Lochter et al., 1997; Page-McCaw et al., 2007). E-cadherin downregulation at cell-cell contacts is involved in EMT and E-cadherin loss has been shown to be causal in the progression of carcinogenesis (Perl et al., 1998). The released ectodomain of E-cadherin following MMP

cleavage promotes invasiveness of cells and detection of high amounts of this fragment is strongly associated with advanced prostate cancer (Noe et al., 2001; Kuefer et al., 2003). It is now crucial to determine whether the likely intracellular end product of these cleavages, E-cad/CTF2, can also influence migration or invasiveness of cells through its signalling role in the nucleus, and whether it also may have a role in tumour progression. It could be speculated that nuclear E-cad/CTF2 could enhance or inhibit the process of EMT, either by acting as a positive signal or by providing a negative feedback mechanism (for example the ICD of γ-protocadherins appears to upregulate the γ-protocadherin gene locus itself (Hambsch et al., 2005)). Alternatively, nuclear E-cad/CTF2 may not affect this process, but have some other role. Insight into the physiological role of nuclear E-cad/CTF2 could be gained by examination of the phenotype of cells expressing E-cad/CTF2 in the nucleus.

It is important to use a system in which nuclear-localised E-cad/CTF2 can be studied in isolation in order to detemine whether any observed phenotypic changes are due to transcriptional changes, since as described in section 6.4.1 (Chapter 6), cytoplasmic sequences of cadherins can act as dominant negatives for cell-cell adhesion. The biological role of E-cad/CTF2 in the nucleus alone needs to be elucidated. MDCK cell lines stably expressing E-cad/CTF2 exclusively in the nucleus have now been established in this study. Although no clear phenotypic change could be detected in these cells upon expression of nuclear E-cad/CTF2 by light microscopy, a more detailed analysis of the phenotype is required. In particular it will be important to measure any changes in migration or invasiveness, and whether there is a difference in how the cells respond to HGF treatment. However p120 seems to be highly involved in E-cad/CTF2's nuclear function in the formation of a complex with DNA and also in its ability to modulate Kaiso-mediated repression. Therefore a cell line with more nuclear and cytoplasmic p120 such as MDA-321 or L-fibroblasts, may be more suitable to observe any phenotypic changes associated with nuclear E-cad/CTF2.

It will also be of vital importance to assess what *in vivo* significance the nuclear localisation of E-cad/CTF2 has. It has been problematic to study the

endogenously produced E-cad/CTF2 following HGF treatment as the amount produced is low relative to the amount of full length E-cadherin. As described in section 1.5.2 and 3.4, the amount of the Notch ICD produced by γ-secretase cleavage is also very small relative to the full length protein. Thus it has not been possible to detect endogenously produced ICDs of either E-cadherin or Notch in the nucleus by immunostaining or nuclear fractionation. Nuclear access of the Notch ICD was demonstrated in *Drosophila melanogaster* embryos in a more sensitive approach by inserting the GAL4-VP16 chimera in the C-terminal region of Notch. This was expressed in embryos that also carry a *Gal4UAS-LacZ* transgene and allowed the detection of expression of the *LacZ* gene in response to Notch ligands, indicating nuclear translocation of the Notch ICD (Struhl and Adachi, 1998). A similar approach could be employed in *Drosophila melanogaster* by fusing GAL4-VP16 to the C-terminus of E-cadherin. This approach could provide insight into where, and at what stage, cleavage and nuclear localisation of E-cadherin occurs during development. It may also be useful to establish E-cadherin mutants that cannot be cleaved by γ-secretase and determine what affect this has on phenotype.

As described in Section 1.5.4 (Introduction), regulation of γ-secretase activity remains incompletely understood. An interesting question to answer is how cleavage of E-cadherin by γ-secretase is upregulated during EMT. It seems very likely that the upregulation of extracellular metalloproteases is involved since as described in section 1.4.5 (Introduction), they are known to be upregulated by HGF, Ras and Snail, and provide γ-secretase with its substrate for cleavage. The findings that γ-secretase-mediated cleavages of both Notch and APP are upregulated by endocytosis (described in section 1.5.4) suggest that this may be a general mechanism for regulating γ-secretase cleavage. In the case of Notch, ubiquitination was shown to be required for endocytosis and cleavage by γ-secretase (Gupta-Rossi et al., 2004). It will be interesting to determine whether endocytosis of E-cadherin which occurs upon HGF treatment of MDCK cells is required for the observed production of E-cad/CTF2. Although it will be difficult to block endocytosis using dominant negative Dynamin or Eps15 due to the low transfection efficiency of MDCK cells, an inhibitor of endocytosis known as

Dynasore has been developed (Thompson and McNiven, 2006). This could be used to determine whether endocytosis is a prerequisite for γ-secretase-mediated cleavage of E-cadherin in this system. Furthermore, it will be interesting to determine whether Hakai can enhance cleavage of E-cadherin since Hakai ubiquitinates and enhances endocytosis of E-cadherin in response to HGF (Fujita et al., 2002). CK1 may also provide a link between endocytosis and cleavage of E-cadherin, since it is known to induce E-cadherin endocytosis (Dupre-Crochet et al., 2007) and it was shown in section 5.5.2 of this thesis that it enhances the nuclear localisation of E-cad/CTF2. It would be interesting to determine whether E-cadherin cleavage is affected in E-cadherin constructs which are mutated at the site of CK1 phosphorylation.

An important finding from this thesis is that E-cad/CTF2 can modulate transcription from reporters containing binding sites for TCF and Kaiso transcription factors. As was expected, E-cad/CTF2 in the nucleus inhibits transcription from the TOP-FLASH reporter in SW620 cells in which β-catenin signalling to TCF/LEF-1 is highly active. This is likely to be due to E-cad/CTF2 competing with TCF/LEF-1 for binding to β-catenin. Furthermore transcription of β-catenin-TCF/LEF-1 gene targets cyclin D1 and myc are also inhibited in SW620 cells. Interestingly, analysis of reporters containing Kaiso binding sites indicated that E-cad/CTF2 in the nucleus can act together with p120 to relieve Kaiso-mediated repression in HEK293 cells. Indeed E-cad/CTF2, p120 and Kaiso form a trimeric complex. E-cadherin and Kaiso both bind p120 within p120's Arm repeat domain, but the data in this thesis suggest that their binding to p120 is not mutually exclusive. Instead the data in sections 6.4.3 and 6.4.4 indicate that E-cad/CTF2 binding to Kaiso via p120 enhances the ability of p120 to relieve Kaiso-mediated repression. Indeed it was also shown that in HEK293 cells, transcription from a reporter construct of the promotor region of a known Kaiso target MMP-7 was enhanced by nuclear E-cad/CTF2 together with p120.

A large number of Kaiso gene targets have both TCF/LEF-1 and Kaiso binding sites within their promotor regions. This may mean that nuclear E-cad/CTF2 could suppress or enhance transcription of these genes depending on the levels of

nuclear β-catenin, nuclear p120 or other signals. Thus it is possible that E-cad/CTF2 in the nucleus may act to balance expression of these genes depending on which signalling pathways are activated. It seems likely that both p120 and β-catenin are released into the cytoplasm when cadherins are downregulated, and thus could be available for nuclear signalling. p120 is stable in the cytoplasm whereas β-catenin is rapidly degraded unless cells are stimulated with Wnt. Therefore in the absence of Wnt, p120 nuclear signalling may be more prominent, and in these situations nuclear E-cad/CTF2 may enhance this. However upon stimulation with Wnt, β-catenin nuclear signalling is activated and under these conditions, E-cad/CTF2 seems to suppress this. To further understand the role of nuclear E-cad/CTF2 regulating the transcription of Wnt/Kaiso target genes, it will be important to examine the effect of nuclear E-cad/CTF2 on transcription of the same targets in the presence and absence of Wnt stimulation. Both ADAM10 and nuclear N-cad/CTF2 have been reported to enhance cyclin D1 expression (Maretzky et al., 2005; Shoval et al., 2007). It is possible that ADAM10 induction of cyclin D1 expression could be partly due to E-cad/CTF2-mediated derepression of Kaiso. Likewise it will be interesting to determine whether N-cad/CTF2 can also regulate Kaiso. There are also specific targets of both TCF/LEF-1 and Kaiso which seem likely to be either repressed or activated by nuclear E-cad/CTF2. For example MTA2, which contains methylated CpG dinucleotides in its promoter, has been shown to be repressed by Kaiso (Yoon et al., 2003). Although p120 has not yet been shown to relieve repression of MTA2, p120 does promote the dissociation of Kaiso from methyl CpG sites (Daniel et al., 2002). The effect of nuclear E-cad/CTF2 on MTA2 expression could be determined by RT-PCR.

Determination of other gene targets of nuclear E-cad/CTF2 will also be of crucial importance to understanding its biological role. The existence of other targets is indicated by the formation of a complex of E-cad/CTF2 with DNA, via p120. It is unlikely that this complex forms at Kaiso-binding sites in DNA, since p120 induces dissociation of Kaiso from DNA (Daniel et al., 2002). These targets could be determined using cells stably expressing nuclear E-cad/CTF2 in microarrays or proteomic arrays. RFP also enhances DNA binding of E-

cad/CTF2, so it is possible that some of the known transcriptional targets of RFP could be regulated by E-cad/CTF2.

There is good evidence that both the downregulation of E-cadherin and upregulation of catenin molecules in the cytosol are involved in developmental processes and cancer progression. As E-cad/CTF2 is produced when E-cadherin is downregulated during EMT, and p120 is involved in its nuclear localisation, determining the physiological role of nuclear E-cad/CTF2 could be of relevance to further understand development and disease.

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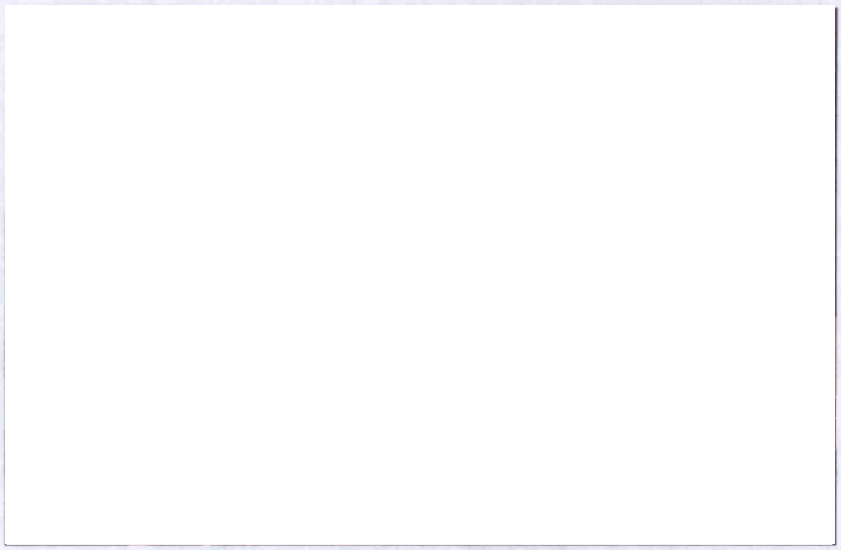

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